Novel Chitosan-Coralline Composite Biomatrices for Bone Tissue Engineering Applications: Microarchitectural Features and *In Vitro* Mesenchymal Stem Cell Responses

Submitted by

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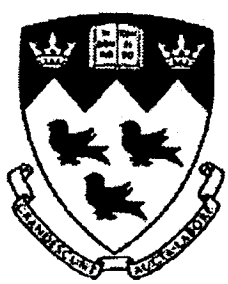

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Engineering.

Your file Votre référence
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To my parents, Marie-Suzanne and Jean-Pierre Gravel,

And

To my fiancé, Mathieu Bélanger.

Words cannot express how grateful I am to you all.

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I wish to express my most sincere gratitude to my supervisor Dr. Maryam Tabrizian, Associate Professor in the Department of Biomedical Engineering at McGill University, for her guidance, invaluable support, and her never-ending availability.

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Table of Contents

LIST OF FIGURES

GLOSSARY OF SYMBOLS

Alkaline phosphatase	ALP
Analysis of variance	ANOVA
Calcium carbonate	$CaCO_3$
Carbon dioxide	CO_2
Extracellular Matrix	ECM
Fetal bovine serum	FBS
Hydroxyapatite	HA
Mesenchymal Stem Cells	MSCs
Microcomputed Tomography	Micro-CT
Phosphate buffered saline	PBS
Polylactide	PLA
Polyglycolide	PGA
Poly(DL-lactic-co-glycolic acid)	PLGA
Scanning electron microscope	SEM
Thermally induced phase separation	TIPS
Three-dimensional	3D
β–Tricalciumphosphate	β–TCP
Weight ratio	wt%

PREFACE

CONTRIBUTION OF AUTHORS

A Thesis submitted to the Faculty of Graduate Studies and Research of McGill University in Partial Fulfillment of the Requirements for the Degree of Master of Engineering.

Thesis background

The research presented in this thesis was performed at the Department of Biomedical Engineering of McGill University under the supervision of Dr. Maryam Tabrizian. The realization of this research was made possible by a scientific exchange between McGill University and the Institute for Applied Biosciences and Department of Biotechnology Engineering of Ben-Gurion University of The Negev, Beer-Sheba, Israel. Through this exchange, a series of visits occurred, ranging in length from a few days for my co-supervisor, Dr. Vago, to approximately two months for his PhD student, Mme Talia Gross, in the course of which some of the more systematic aspects of the research *in vitro* were carried out. She particularly contributed to test methods for *in vitro* assay optimisation and standardisation.

The aim of this study was to develop novel 3D coralline-chitosan macroporous composite scaffolds for tissue engineering with improved mechanical and biological properties in order to improve the *in vitro* stage of the bone tissue engineering strategy.

This work was funded by Ministère du Développement Économique et Régional et de la Recherche (MDERR) Fonds Québécois de Recherche sur la Nature et les Technologies (FQRNT)-regroupment stratégique, and by the Natural Sciences and Engineering Research Council of Canada (NSERC).

Thesis outline

This thesis has been entirely written by the candidate, in collaboration with her supervisor, Dr.Tabrizian, and co-supervisor, Dr.Vago. It consists principally of a

literature review and two journal papers to be submitted. The papers are based on data collected and analyzed by the candidate, with the help of Mme Gross during her visit between July 2nd and August 25th, 2003. As a consequence, in the authorship list of both papers, the name of the candidate appears first, as the primary contributor, whereas the second author, although scientifically and technically involved, had a lesser contribution as collaborator. The third author is the co-supervisor and finally, the fourth is the director of the research.

The thesis is prepared in a "manuscript-based" format and is organised in six chapters. A reference list is included for each chapter.

Chapter 1 describes the research topic and it's rational.

Chapter 2 states the objectives of the research.

Chapter 3 provides a general review of the rational behind the present master's project. It first introduces the reader to the concept of bone tissue engineering. Then, the importance of scaffolding design, material properties and novel method is detailed, underlining the need for new combinations of materials and the future direction. The advantages of composite scaffolds and the factors influencing their properties are highlighted. The characteristics of synthetic and natural polymers as well as calcium-based materials are discussed. Finally, the rational behind using chitosan polymer and coral bioceramic for the development of new composite scaffolds is covered and their appropriateness for bone repair and regeneration is discussed.

Chapter 4 (Paper 1) discusses findings from the development of the novel 3D coralline-chitosan macroporous composite scaffolds for bone tissue engineering, which have formed the subject of a paper that has been accepted for publication in the journal "Tissue Engineering" entitled: "Use of Natural Coralline Biomaterials as reinforcing and foaming agent for developing novel hybrid biomatrices: Microarchitectural and mechanical studies". It describes and contrasts the fabrication process of the scaffolds, in which the

coral was simultaneously used as particulate reinforcing phase and gas-forming agent. It also discusses the effects of the coral:chitosan weight ratio parameter on the physical properties of the scaffolds and the preliminary results of the cell-material interaction observed. This manuscript proposes a new family of coralline-chitosan composite scaffolds with improved mechanical and biological properties for *in vitro* bone tissue engineering.

Chapter 5 (Paper 2) presents the results obtained from the evaluation of the cellular responses to these novel coralline-chitosan macroporous composite scaffolds. These results have been reported in a manuscript that has been accepted for publication in the journal of "Biomaterials". This manuscript is entitled: "Responses of Mesenchymal Stem Cell to Chitosan-Coralline Composites Microstructured Using Coralline as Gas Forming Agent". It discusses findings from the investigation of their supportive activity of cellular attachment, proliferation and differentiation. It also discusses the effect of cell culture medium supplementation with β-glycerophosphate and dexamethasone on the osteogenic phenotype in relation with the type of scaffolds on which the cells are living. This paper ascertains the findings of the previous paper, and further suggests that coralline-chitosan composite scaffolds, especially with high coralline ratios, might enhance the proliferation and phenotype expression of osteoblasts, in comparison with pure chitosan scaffolds.

Chapter 6 summarizes and interprets the general findings of the study and some future researches are suggested.

ABSTRACT

The purpose of this work was to develop novel 3D coralline-chitosan macroporous composite scaffolds for *in vitro* bone tissue engineering and investigate cellular responses to these scaffolds. In these composites, coral skeletal material, which is made of calcium carbonate ($CaCO_3$), was simultaneously used as particulate reinforcing phase and gas-forming agent to obtain a structure with large pores and improved mechanical and biological properties. The reaction between the coralline material and the acidic chitosan polymer solvent, which produces carbon dioxide (CO_2), was rapidly stopped by the subsequent thermally induced phase separation step, leaving coral particulates in the polymeric structure. Scaffolds containing 5 different proportions of coralline material (0, 25, 50, 75, and 100 wt%) were developed and studied under two different aspects. In a first part, the coralline:chitosan weight ratio parameter was studied for its effects on the physical properties of the scaffolds with a combination of scanning electron microscope (SEM), micro-CT imaging, and compression testing. In a second part, the scaffolds were cultured with mice MSCs. Cellular morphology, DNA content, as well as expression of osteogenic markers alkaline phosphatase (ALP) activity and osteocalcin release were evaluated. The effect of cell culture medium supplementation with β-glycerophosphate and dexamethasone was studied. The results showed that higher coralline concentration increased the pore wall thickness and favoured large pore formation. Varying the coralline powder to chitosan polymer ratio from 0 to 75 wt% increased the observed pore sizes from 80 μm to 400 μm in average and decreased the porosity from 91% to 78%. The equilibrium compressive modulus was improved proportionally with the coral content, and the 75 wt% composites had a significantly higher modulus than all the other chitosan-based scaffolds. The corallline scaffolds showed by far the highest evaluation of cell number and ALP activity over all the other chitosan-based scaffolds. They were the only materials on which the osteocalcin protein was release throughout the study and generally at a high level. Nevertheless, the coralline:chitosan composite scaffolds containing high coralline ratios generally showed higher results than the pure chitosan scaffolds. Of all the

chitosan-based scaffolds, the cells cultured on the 75:25 coralline:chitosan scaffolds obtained the highest peak of ALP activity and generally obtained the highest cell number. While the presence of osteogenic supplements had no obvious effect on cell behaviour and osteogenic differentiation, distinct cell morphology and osteoblastic phenotype expression were observed depending on the coralline to chitosan ratios composing the scaffolds. The results strongly suggest that coralline:chitosan composites, especially those having a high coralline content, may enhance adhesion proliferation and osteogenic differentiation of MSCs in comparison with pure chitosan. In conclusion, composite scaffolds with improved mechanical and biological properties concomitant with large pores were achieved by increasing the coralline:chitosan weight ratio. These composites possess therefore many advantages over coralline and chitosan scaffolds, suggesting that they have an excellent potential as biomatrices for tissue engineering.

RÉSUMÉ

Cette étude fut accomplie dans le but de développer une famille de composites fait de chitosan et de corail servant de matrices tridimensionnelles pour l'ingénierie tissulaire de greffes osseuses. Lors de la confection de ces composites, le corail, fait de calcium carbonate ($CaCO_3$), fut utilisé à la fois comme agent de renforcement et de gonflage pour obtenir une structure composée de larges pores et ayant des propriétés mécaniques et biologiques améliorées. Lors de la première étape du développement, une réaction entre le corail et le solvant acide du chitosan en solution a libéré du gaz carbonique. Cette réaction fut arrêtée très rapidement, lors de la congélation, qui fut suivie de la lyophilisation, laissant les particules de corail dans la structure de polymère. Des matrices contenant 5 proportions différentes de corail (0, 25, 50, 75, and 100 wt%) furent développées et étudiées sous deux aspects. Dans un premier temps, l'effet du ratio corail:chitosan sur les propriétés physiques des matrices composites fut évaluée à l'aide de microscope électronique à balayage (SEM) et de micro-CT en combinaison avec des tests de compression mécaniques. Dans un deuxième temps, les matrices furent cultivées avec une lignée cellulaire mesenchymal souche de souris. La morphologie cellulaire, le contenu d'ADN, et l'expression de marqueurs de différenciation ostéoblastiques, mesurée par l'activité enzymatique de ALP et la libération d'osteocalcine furent évalués. De plus, l'influence sur les cellules de l'ajout au milieu de culture de suppléments ostéogéniques, β-glycerophosphate et dexamethasone, fut étudié. Les résultats démontrent que plus la concentration de corail augmente, plus les parois s'élargissent et plus la formation de larges pores est favorisée. En augmentant la concentration de corail de 0% à 75%, la largeur des pores observée semble avoir passé d'environ 80 μm à 400 μm et la porosité a diminué de 91% to 78%. Il fut noté que le module de compression à l'équilibre a augmenté de façon proportionnelle à la concentration de corail, et que les matrices contentant 75wt% de corail ont un module de compression à l'équilibre significativement plus élevé que toutes les autres matrices de chitosan contenant ou non du corail. Les résultats de la culture cellulaire semblent prometteurs. Globalement, il fut observé que les

matrices de corail ont obtenu un rendement de loin supérieur à toutes autres matrices étudiées en terme de nombre total de cellules et d'activité enzymatique de l'ALP. De plus, la matrice de corail est le seul substrat sur lequel les cellules ont libéré l'ostéocalcine tout au long de l'étude. Néanmoins, les matrices composites de corail-chitosan ayant un ratio de corail élevé ont obtenu généralement des résultats plus élevés que les matrices de chitosan pure. Parmi toutes les matrices contenant du chitosan, les cellules évoluant sur les matrices composées à 75 wt% de corail ont obtenues le plus hauts pic d'activité ALP, et ont généralement obtenu le plus grand nombre total de cellules. En comparaison avec les matrices de pure chitosan, les matrices composites de coral-chitosan semblent mieux performer, avec une meilleure affinité cellulaire, en stimulant la formation de nombreux pseudopodes et filopodes et une adhésion cellulaire plus rapide. En conclusion, il fut observé que l'augmentation de la concentration de corail dans les matrices composites de chitosan résulte en une structure poreuse, renforcée, et ayant de larges pores. En tenant comptes des limites de la présente étude *in vitro*, les résultats obtenus suggèrent que la présence de suppléments ostéogéniques dans le milieu de culture ne semble pas avoir affecté le comportement cellulaire de façon précise. Cependant, cette étude suggère fortement que les matrices composites de corail-chitosan, particulièrement celles contenant une grande concentration de corail, puissent encourager l'adhésion, la prolifération et la différentiation ostéoblastique, en comparaison avec les matrices de chitosan pure.

CHAPTER 1: INTRODUCTION AND RATIONALE OF THE RESEARCH

Bone tissue engineering offers one of the most promising alternative approaches to the actual bone transplantation [1,2]. One of the novel strategies for engineering bone tissue is to use a hybrid biomaterial, formed from a combination of undifferentiated culture-expanded osteogenic cells obtained from patients, seeded onto an appropriate temporary scaffold, *in vitro*, in the presence of osteogenic supplements [3-6]. These conditions induce the cells to differentiate toward the osteoblast lineage, before being transplanted back into the same patient [7-9]. The exposed cells could then become active osteoblasts forming bone matrix within the three-dimensional porous structure of the scaffold [4], which mimics the natural extracellular matrix [10]. It then gradually degrades, as new tissue is formed [11,12].

Since connective tissues are anchorage dependent [10,13], the porous scaffold is needed to (i) provide a temporary mechanical support, (ii) guide cell attachment, growth, and tissue formation and organization in 3-dimensions, (iii) and promote tissue regeneration [14,15].

Consequently, the design characteristics and the selection of scaffolding biomaterials can significantly affect the whole development of engineered bone tissues [16]. Therefore, an ideal scaffolding material for engineering bone tissue must satisfy a number of requirements, namely it must be highly porous with an interconnected pore network; be biocompatible and bioresorbable with an adequate surface chemistry to provide an appropriate regulation of cell behaviour such as cell attachment, proliferation, migration and differentiation, and possesses a degradation and resorption kinetics that match tissue formation *in vitro* and/or *in vivo* to maintain its structural integrity, and it must be easily processed into desired shapes [11,14,17,18,19]. Futhermore, the key to regenerate bone

successfully is also to provide the host site with sufficient osteoprogenitor cells delivered in a suitable scaffold insuring osteoblastic differentiation [20].

Finding such material is a major challenge since no homogeneous material currently available provides these essential features [1,19,21]. A logical approach is to design a composite that combines the favourable properties of each phase while minimizing the shortcomings of homogenous scaffolds [1,22].

Several types of biological tissues occur in nature as composites, such as skeleton, teeth or shells of organisms. They are made from organic and inorganic phases, which play specific roles and together fulfill the mechanical and biological properties required in their particular functions [1]. In bone, collagen and other noncollagenous proteins are associated with inorganic bone mineral, mainly hydroxyapatite. Similarly, in crab shell, chitin is combined with calcium carbonate [1,23,24].

To obtain such a composite, many researchers have recently developed macroporous composite scaffolds made of a chitosan matrix reinforced with bioceramics particulates filler [5,11,25-28] to fabricate macroporous composite scaffolds with reinforced matrices and improved bioactivity [17]. However, although the composite scaffolds investigated have improved properties, it seems that none of them have achieved the ultimate goal of bone tissue engineering: to create a device that has the capacity to replace autologous cancellous bone for the management of bony defect [29]. The development of adequate biodegradable scaffolds is still a main issue that needs to be resolved [21].

Consequently, we propose here to develop novel coralline:chitosan composite scaffolds with improved mechnical and biological features. Both chitosan and coralline material are reported to be excellent candidates for bone repair and regeneration. The combination of their favourable properties may further enhance tissue regenerative efficacy. Moreover, in such composite, while chitosan would be providing a form, coral skeletal material is expected to act simultaneously as reinforcing particulates as well as gas-forming agent. The incorporation of coral powders into chitosan polymers would result in macroporous

composite scaffolds with reinforced microstructure, large pore sizes and improved biological properties.

REFERENCES

1. Laurencin CT, Lu H.H. Polymer-Ceramic Composites for Bone-Tissue Engineering. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 462-468.
2. Ishaug-Riley SL, Crane-Kruger GM, Yaszemski MJ, Mikos AG. Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. Biomaterials. 1998 Aug;19(15):1405-12.
3. Yaszemski MJ, Oldham JB, Lu L, Currier BL. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 541-547.
4. Ohgushi H, Caplan AI. Stem cell technology and bioceramics: from cell to gene engineering. J Biomed Mater Res. 1999;48(6):913-27.
5. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. Nat Biotechnol. 2000 Sep;18(9):959-63.
6. Fricain JC, Bareille R, Ulysse F, Dupuy B, Amedee J. Evaluation of proliferation and protein expression of human bone marrow cells cultured on coral crystallized in the aragonite of calcite form. J Biomed Mater Res. 1998 Oct;42(1):96-102.
7. Khan Y, Pratt LT, Laurencin CT. Bone Graft Substitute Materials. emedicine. 2004 mar 3.
8. Gao J, Dennis JE, Solchaga LA, Awadallah AS, Goldberg VM, Caplan AI. Tissue-engineered fabrication of an osteochondral composite graft using rat bone marrow-derived mesenchymal stem cells. Tissue Eng. 2001 Aug;7(4):363-71.
9. Yoshikawa T, Ohgushi H, Akahane M, Tamai S, Ichijima K. Analysis of gene expression in osteogenic cultured marrow/hydroxyapatite construct implanted at ectopic sites: a comparison with the osteogenic ability of cancellous bone. J Biomed Mater Res. 1998 Sep 15;41(4):568-73.

10. Risbud M. Tissue engineering: implications in the treatment of organ and tissue defects. Biogerontology. 2001;2(2):117-25.
11. Zhang Y, Ni M, Zhang M, Ratner B. Calcium phosphate-chitosan composite scaffolds for bone tissue engineering. Tissue Eng. 2003 Apr;9(2):337-45.
12. Ma PX, Choi JW. Biodegradable polymer scaffolds with well-defined interconnected spherical pore network. Tissue Eng. 2001 Feb;7(1):23-33.
13. Zhang R, Ma PX. Porous poly(L-lactic acid)/apatite composites created by biomimetic process. J Biomed Mater Res. 1999 Jun 15; 45(4): 285-93.
14. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues-state of the art and future perspectives. J Biomater Sci Polym Ed. 2001;12(1):107-24.
15. Hollister SJ, Maddox RD, Taboas JM. Optimal design and fabrication of scaffolds to mimic tissue properties and satisfy biological constraints. Biomaterials. 2002 Oct;23(20):4095-103.
16. Godbey WT, Atala A. *In vitro* systems for tissue engineering. Ann N Y Acad Sci. 2002 Jun; 961: 10-26.
17. Zhang Y, Zhang M. Three-dimensional macroporous calcium phosphate bioceramics with nested chitosan sponges for load-bearing bone implants. J Biomed Mater Res. 2002 Jul; 61(1): 1-8.
18. Temenoff JS, Lu L, Mikos AG. Bone-tissue engineering using synthetic biodegradable polymer scaffolds. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp.454-459
19. Gunatillake PA, Adhikari R. Biodegradable synthetic polymers for tissue engineering. Eur Cell Mater. 2003 May 20;5:1-16.
20. Zhao F, Yin Y, Lu WW, Leong JC, Zhang W, Zhang J, Zhang M, Yao K. Preparation and histological evaluation of biomimetic three-dimensional hydroxyapatite/chitosan-gelatin network composite scaffolds. Biomaterials. 2002 Aug;23(15):3227-34.
21. Khan SN, Tomin E, Lane JM. Clinical applications of bone graft substitutes. Orthop Clin North Am. 2000 Jul;31(3):389-98.

22. Wang M. Developing bioactive composite materials for tissue replacement. Biomaterials. 2003 Jun;24(13):2133-51.
23. Muzzarelli C, Muzzarelli RA. Natural and artificial chitosan-inorganic composites. J Inorg Biochem. 2002 Nov 11;92(2):89-94.
24. Wang X, Ma J, Wang Y, He B. Structural characterization of phosphorylated chitosan and their applications as effective additives of calcium phosphate cements. Biomaterials. 2001 Aug;22(16):2247-55.
25. Zhang Y, Zhang M. Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. J Biomed Mater Res. 2001 Jun 5; 55(3): 304-12.
26. Lee YM, Park YJ, Lee SJ, Ku Y, Han SB, Choi SM, Klokkevold PR, Chung CP. Tissue engineered bone formation using chitosan/tricalcium phosphate sponges. J Periodontol. 2000 Mar;71(3):410-7.
27. Zhang Y, Zhang M. Calcium phosphate/chitosan composite scaffolds for controlled *in vitro* antibiotic drug release. J Biomed Mater Res. 2002 Dec 5;62(3):378-86.
28. Ge Z, Baguenard S, Lim LY, Wee A, Khor E. Hydroxyapatite-chitin materials as potential tissue engineered bone substitutes. Biomaterials. 2004 Mar;25(6):1049-58.
29. Linhart W, Peters F, Lehmann W, Schwarz K, Schilling AF, Amling M, Rueger JM, Epple M. Biologically and chemically optimized composites of carbonated apatite and polyglycolide as bone substitution materials. J Biomed Mater Res. 2001 Feb;54(2):162-71.

CHAPTER 2: THESIS OBJECTIVES AND OUTLINE

This thesis aimed at developing novel three-dimensional (3D) macroporous coralline:chitosan composites scaffolds for bone tissue engineering. The research hypothesis was driven from the excellent properties of both chitosan and coral as biomaterials, and the evidence of their biomedical potential as homogeneous material proven through a large number of researches in the field. The novelty of this work relies on the first attempt to use both chitosan and coral in the development of macroporous biodegradable composites and on the introduction of an innovative method of scaffolds fabrication. Composite matrices with different proportions of chitosan and coral were prepared and studied. Firstly, the scaffolds were examined ultrastructurally, and secondly their ability to regulate the cellular activity has been assessed.

To fulfill the aim of this thesis, the following objectives have been achieved:

1. Chitosan-based hydrogel scaffolds containing three different ratios of coralline material (25, 50, and 75 wt%) were prepared along with pure coral and pure chitosan scaffolds as control materials.

2. To assess the relationship between scaffold composition, microarchitecture and mechanical properties, a combination of SEM, and micro-CT imaging, were used along with compression tests using Mach-1 mechanical testing.

3. To study the effect of the scaffold physical characteristics on cell behaviour, the scaffolds were seeded with mice MSCs ORL-12525, and analyzed for their ability for cellular attachment, proliferation and differentiation.

    a) Cell morphology on the scaffolds was observed through SEM.
    b) Cell proliferation was assessed with DNA cyquant.

c) Cell differentiation was evaluated by the expresssion of phenotypic markers of osteoblastic differentiation: ALP activity and osteocalcin release.

4. The effect of cell culture medium supplemented with β-glycerophosphate and dexamethasone on the osteogenic phenotype of these cells was studied in relation with the type of scaffolds.

CHAPTER 3: LITERATURE REVIEW

TISSUE ENGINEERING AND COMPOSITE BIOMATERIALS

3.1. CLINICAL NEEDS

Each year, there are about 6.5 millions fractures happening in the United States. 15% of these fractures are difficult to heal or problematic either because the healing is slow (delayed union), does not occur (non-union) or occur in a deformed position (mal-union) [1]. In addition to fractures, poor bone healing occurs in a wide variety of clinical situations including trauma and disease. This situation often reduces quality of life with chronic pains and reduces mobility over longer periods and may require surgery. This has a considerable socioeconomic impact [1,2].

The treatment of such posttraumatic skeletal conditions and other problems of extensive local bone loss is a significant clinical challenge [3,4]. External fixation devices that restore the alignment and stabilize bones at risk of poor healing may be all that is necessary to achieve a successful reconstruction [1,3]. However, in many cases, adjunctive measures are required [3], of which the most common therapy performed by clinicians is the augmentation of critical-sized defects and non-healing fractures with bone grafting procedures [1]. By filling defects or cavities in bone, these bone grafts are often necessary to enhance biologic repair of skeletal defects because they provide support, promote fusion, and help the body regenerate new bone [3,5,6].

Consequently, bone is the most commonly replaced tissue of the body with an estimated 500,000 to 1,000,000 bone grafting procedures done annually in the United States [7,8,9]. However, because it is an active living tissue and performs several functions, loss bone is very difficult to replace adequately [10].

3.2. CURRENT OPTIONS

Current treatment of bony defect involves four basic strategies: autografts, allografts, xenografts, and bone graft substitutes [11]. These graft materials all have their own advantages and disadvantages and can be used alone or in combination with each other [4,12]. The choice of the appropriate graft material must be a function of the requirements of the intended application, the graft availability, and its cost [3]. Autografts and allografts are the main strategies used and represent 90% of the overall bone graft market [7,13]. The 10% left of the bone graft market is represented by the bone graft substitutes. However, their share is increasing, as familiarization, clinical experience and confidence in their use as safe and effective grafts are accrued [7,13]. The use of xenografts, procured from other species, is not common [11].

3.2.1. Autografts

Autologous bone grafts, which is the bone taken from another part of the patient's own body, usually the iliac crest, is the clinically preferred grafting materials for bone replacement. The advantages are their excellent success rate of 80% to 90%, and a low risk of transmitting diseases and histocompatibility [3,14]. Autogenous bone grafts possess the following three essential elements for healing that an ideal bone grafting material should provide and against which all bone graft substitutes can be compared [3,9,15,16].:

1. An osteoconductive structural matrix to provide physical support on which bone cell can attach, migrate, grow and divide, and where vascular and cellular infiltration can occur.
2. Osteoinductive factors, generally proteins that have the ability to induce differentiation of pluripotent stem cells to osteogenic cells or induce stem cells to proliferate.
3. Osteogenic cells or undifferentiated stem cells that are capable of differentiating and lay down new bone matrix.

Currently, trabecular bone autograft is the gold standard autograft material in the treatment of bony defects for the following reasons [17]: although cortical bone may be preferred if immediate biomechanical support is needed from the bone graft, it has a low surface limiting osteoconduction, and a minimum of osteogenic elements [3,9,18]. On the other hand, cancellous bone possesses excellent osteoconductive porous structure composed of a trabecular network of small branching bony spicules or trabeculae, which provide a large surface area for bone formation and contains a greater proportion of osteoinductive and osteogenic elements than compact bone [9,14]. Consequently, cancellous bone is not immediately mechanically supportive, but instead, has the more significant capacity of delivering bone-matrix proteins and cells to the host site and become incorporated more quickly and completely [19]. There are three important differences in the way autologous cancellous and cortical transplants are incorporated:

1. Revascularization through open pores of trabecular bone is done much faster than through Haversian canals of compact bones, 2 weeks compared to 2 months [20].
2. The osteoblastic response to a trabecular graft results in the formation of new woven bone on its surface with no prior resorption of the trabeculae and allows a fast incorporation and a regain of the same strength as cortical graft over a period of 6 to 12 months. Whereas due to the initial osteoclastic host response to cortical bone, the graft is resorbed and becomes weaker, possibly loosing 50% of its strength in 6 months before the osteoblasts make new woven bone, and allow the graft to regain initial strength after 1-2 years [3,9,20].
3. Finally, cancellous transplant is totally resorbed and transformed in lamellar bone in the remodelling phase, while the cortical bone graft may never be completely resorbed, and necrotic tissue may remain there for years, which may not allow optimal mechanical properties [20].

Nevertheless, it is well documented that there are significant limitations and complications from the use of autografts. They are limited in supply, need a second surgical procedure associated with chronic donor site pain and potential donor site morbidity and require more hospital time than allograft or bone-graft substitutes [3,5,6].

3.2.2. Allografts

Given these limitations associated with autograft, allograft is the next best alternatives at present time [16]. Allografts are harvested from donors or cadavers. They can be used to fill small defects but are especially attractive for reconstruction after major bone loss. Allograft bones provide immediate structural support, and are osteoconductive [3,5]. Though allografts and xenografts are attractive sources, their use encounters serious concerns. They have been reported to have a significant incidence of postoperative infection and fracture as well as the possibility of disease transmission, histoincompatibility, loss of biological and mechanical properties once processed and low availability due to financial and religious concerns [3-6].

3.2.3. Bone graft substitutes

Despite the benefits of autografts and allografts, the limitations of each have prompted increasing interest in alternatives and have led, during the past 30 years, to the development of several bone graft substitutes [2,4,21].

Bone graft substitutes can replace, extend or expand a certain amount of autologous bone graft [3]. Thereby, they avoid or reduce the need for the removal of the patient's own bone, sparing the patient pain and morbidity and are especially useful for patients for who related inherent risks of a harvesting procedure are too high [5].

Other benefits include their easy sterilization and storage [4,7]. Consequently, they have lower rejection and infection rates than allografts. Moreover, their unlimited supply make them available at a consistent quality, unlike allografts, for which availability may be restricted because of increasing surgical procedures requiring more bone graft material than allograft donors can provide [5].

Bone graft substitutes that are biodegradable and bioresorbable are desirable in many clinical applications. They have the advantages of being incorporated in the recipient site, degraded and further resorbed, leaving no foreign material in the body and increasing the

available space for new bone regeneration [18,22,23]. This new bone, called woven bone, can then remodel in response to load bearing into the structurally more organized lamellar bone. Through this remodelling process, the mechanical properties of the bone-biomaterial composite are enhanced, and later on, a functional mature bone, either trabecular or compact bone, can be completely regenerated [18,20,24,25]. Therefore, eventual graft biodegradation reduces any phenomenon of "stress shielding", and eliminates the risks of potential complications associated with permanent bone implants, such as infection [18].

A range of bone graft substitutes has been developed, varying in composition and characteristics [25,26]. There are now several biodegradable substitutes for bone grafting from synthetic or biological origin now commercially available [27]. The most important of these, based on natural materials, are demineralized human bone matrix, bovine collagen mineral composites, marine corals and coralline hydroxyapatite, and those based on synthetic materials are calcium sulfate pellets, bioactive glass, and synthetic calcium phosphates, especially tri and tetracalcium phosphates or hydroxyapatite [25,28-32].

All substitute materials for bone grafting share several advantages over autogenous and allogenous bone grafts [7,16]. However, although some of these substitutes are bioactive and osteoconductive, they do not intrinsically possess all the properties of bone autografts, i.e. they lack osteogenicity and do not induce bone formation. Moreover, their success in repairing massive bone defects is limited, and is still a great challenge to reconstructive surgery [33,34]. Therefore, despite the many advances in bone graft substitutes, there has not been a single ideal substitute and the search for the perfect solution still continues [26,35]. Significant efforts are being made in the research for new biomaterials and approaches to heal skeletal defects, in order to develop biological alternatives that will circumvent the limitations associated with existing bone graft substitutes, and enhance their functional capacities [26,35,36].

3.3. BONE TISSUE ENGINEERING STRATEGY

Bone-tissue engineering has emerged as one of the most promising alternative approach to actual bone grafts, offering the option of viable autogenous bone grafting systems with all the advantages and without being subjected to the limitation of supply and donor sites morbidity [36,37].

The fundamental goal of tissue engineering is to repair, restore, or regenerate natural tissues and their function by applying biological, chemical, and engineering principles to the development of biological substitutes whose function mimics that of natural tissue, using biomaterials, cells and factors alone or in combination [26,36]. Although many other approaches may be investigated, several laboratories worldwide are focusing on the strategy to engineer bone-tissue that mimics natural cancellous bones as potential alternative to the patient's own [17,19]. This may overcome an expanding need for autogenous cancellous bone grafts given their excellent properties, high efficacy and successful results in bone regeneration.

The first objective is to provide the right carrier, and since scaffold 3-dimensional structure defines the ultimate shape of the regenerating tissue [23,38], the scaffold needs to have a morphology analogue to that of cancellous bone. Similarly, such scaffold would be advantageous, having an osteoconductive structure that acts as cells delivery mechanism once seeded with autologous, potentially osteogenic cells.

In fact, one of the novel strategies for engineering bone tissue is the use of a hybrid biomaterial, formed from a combination of undifferentiated culture-expanded osteogenic cells obtained from patients, seeded onto an appropriate temporary scaffold, *in vitro*, in the presence of osteogenic supplements [33,34,39,40]. These conditions induce the cells to differentiate toward the osteoblast lineage, before being transplanted back into the same patient [26,41,42]. The exposed cells can then become active osteoblasts, forming bone matrix within the three-dimensional porous structure of the scaffold [40]. Then, it gradually degrades as new tissue is formed [43,44]. When implanted *in vivo*, these cells

survive and subsequently such *in vitro* prefabricated bone continues to form new bone [40].

In this composite, an embryonic environment is recreated where osteogenic cells can both proliferate and differentiate on the biomaterial surface. These two steps are required in order to have a good biointegration and functional bone substitution, once implanted, and may thus improve clinical outcome [33,34,45].

The *in vitro* tissue-engineered construct will be structurally inferior to normal trabecular bone until it is fully accommodated and remodelled by the host tissue [23,46]. Once implanted *in vivo*, controlling the mechanical environment of the bone defect in part with internal or external fixation devices is more suitable [46]. This way, the tissue transplant is not submitted to excessive forces, its structural integrity is maintained, but it can still bear some load. Thus, in response to that load, it can be slowly remodelled by the host into a more organized lamellar bone that will completely match the bone structurally and mechanically and assume its structural role [20,23,46,47].

Yet, it is quite improbable that this trabecular-like engineered tissue would routinely become the solution to the reconstruction of massive, load-bearing bone defects [47]. Instead, unlike the rationale of grafting compact bone, where the biomechanical strength is the key requirement [19], this strategy exploits the rapid and effective ability of the engineered construct to regenerate small or medium size bony defects [47].

3.4. MSCs

Patient-derived osteoblasts or osteoblasts precursor cells are potential sources for autologous skeletal tissue engineering that can be derived either from periosteum or bone marrow [3,48,49]. Bone marrow-derived mesenchymal stem cells (MSCs), are known to be the most practical and suitable source, having the advantages of being easily harvested and cultured *in vitro* [42]. Compared to the osteogenic osteoblasts and periosteal cells, MSCs can be simply harvested by needle aspiration of the bone marrow [42]. MSCs are a

pluripotent population, having the potential to differentiate into bone, cartilage, muscle, tendon, and other connective tissues [45,48,50]. Although MSCs are rare progenitor cells, approximately 1/100,000 nucleated cells in adult bone marrow, once purified, they can be mitotically expanded through *in vitro* cell culture technology to almost any extent, over one billionfold, without loss of developmental potency [40,51]. *In vitro* expanded autologous MSCs are now used because faster and more uniform bone formation occur on the material surface [34,40]. Furthermore, inducing their differentiation *in vitro* before implantation, results in even faster tissue repair [40].

3.5. OSTEOBLASTIC DIFFERENTIATION *IN VITRO*, STAGES AND ASSOCIATED MARKERS

Although the number of steps involved in osteoblastic differentiation is not known [52], at least three distinct stages of cellular activity can be observed *in vitro*: proliferation, extracellular matrix maturation, and matrix mineralization [53,54]. These progressive changes may be associated with temporal acquisition of biochemical properties, such as ALP activity, and expression bone matrix-associated proteins, such as osteocalcin, which thus, currently provide a guide for defining events of osteodifferentiation [54].

ALP is a cell surface protein tough to be involved in transmembrane signalling, in the regulation of proliferation, migration, and differentiation in osteoblastic cells and even in the mineralization process [53]. In mineralized bone-tissue, osteocalcin represents less than 15% of the non-collagenous bone-matrix proteins [54]. It is tough that osteocalcin regulates mineral maturation and that it may mediates bone resorption, but all its implications in bone formation and remodelling remain to be elucidated [54].

Although useful, the osteoblasts-associated markers cannot be related precisely with stages of differentiation [52,54]. However, there are generalized paradigms: in general, early osteoprogenitors are highly mitotic cells expressing collagen I and ALP soon after plating [54]. As proliferation decreases and pre-ostoblasts differentiate into osteoblasts, ALP expression increases and then decreases when mineralization is well progressed

[55]. Osteocalcin expression increases with mineral deposition, and once the mineralization has been initiated, is detected at a high level that is further maintained as osteoblast differentiation progress into osteocytes and/or lining cells [54].

Therefore, while ALP and Collagen I synthesis occurs early in the differentiation process and are characteristic of the osteogenic lineage, osteocalcin is induced in fully differentiated osteoblasts and is thus a very late marker of osteoblast maturation more specific to bone and mineralized connective tissues [53,54].

3.6. FACTORS INFLUENCING CELL PROLIFERATION, AND DIFFERENTIATION

In vitro, particular environmental or local conditions, including (i) the presence of soluble inducers, (ii) cell-cell interactions, and (iii) cell-matrix interactions, have been proposed to be important factors for directing tissue formation [53,54]. These extracellular stimuli are believed to influence the response of osteoblastic lineage cells such as proliferation, differentiation, and metabolic activity [53,54,56].

3.6.1. Soluble inducers / Effect of the osteogenic supplementation

Exposing the undifferentiated cell in culture to osteogenic supplements such as ascorbic acid, β-glycerophosphate, and dexamethasone, directs the mesenchymal stem cell's differentiation towards the osteoblast lineage [26,40,57]. *In vitro*, while ascorbic acid enhances collagen synthesis, β-glycerophosphate supplies the phosphorus necessary for bone matrix formation and thus promotes mineralization [58]. Dexamethasone, a synthetic glucocorticoid, has been demonstrated to induce osteoblastic differentiation [57-63].

It was suggested that glucocorticoids, notably dexamethasone, would be absolutely required for osteoprogenitor cell differentiation in rodent marrow stromal cultures [57,62]. In contrast, some other studies reported that dexamethasone was not essential,

but that it was increasing the osteoblast-associated markers expression including ALP and osteocalcin [64].

Conflicting results on the influence of glucocorticoids on bone cells have been observed and are far from being well understood. The effects of dexamethasone observed *in vitro* appeared to vary with the concentration, time and duration of treatment [63-66], the characteristics of the donor and the location in the skeleton from where the cells are harvested [60,67], the cell seeding density and the maturation of the cells studied [63,64,66].

3.6.2. Cell-cell interactions

Numerous studies strongly support an important role of cell-cell interaction in osteoblast differentiation and that it may be essential for the coordination of matrix production and mineralization [67-70]. Cell-cell interactions are mediated especially by cadherins, which are a family of cell surface adhesion receptors forming adherence junctions once the cells have adhered and interact with each other [67]. Although their role in osteoblast function remains to be elucidated, previous observations suggest that osteoblast cadherins may be directly involved in osteoblastic differentiation [68], to induce the expression of ALP and to be necessary for the mineralization [67,68,70]. Furthermore, cadherins are known to mediate the formation of gap junction, which allow for communication between neighbouring osteoblastic cells cytoplasm [70]. The inhibition of gap junction was reported to decrease the ALP activity, mineralization, and osteocalcin expression [69].

Cell seeding strategy is therefore believed to be an important determinant in osteoblastic differentiation *in vitro*. It was previously demonstrated that increased osteoprogenitor cell seeding density was related to higher degree of matrix mineralization [64,71,72].

3.6.3. Cell-matrix interactions

In order to survive, many anchorage dependant cells, such as MSCs and osteoblasts, require a supportive matrix that must allow for their attachment [73,74]. In addition to cell viability, it is thought that the initial cellular attachment regulates the cell shape and behaviour such as growth, movement, and ultimately differentiation of cells [73,75].

Cellular adhesions occur at the cell-material interface through "ruffled membranes", focal adhesions, adhesion plaques or focal contacts [56,76]. Focal contacts are discrete regions of a cell membrane, approximately 0.1-2 μm wide and 2-10 μm in length, binding to the external environment via integrin receptors [76]. The signal detected by these receptors is transferred through a complex of different molecules to actin filaments in stress fibres, responsible for the contractile mechanisms of the cell [77], and hence affects the cytoskeleton and the cell shape [56,76]. The signal is also further transduced via the cytoskeleton to the nucleus, resulting in new gene transcription and expression of specific phenotypes [56,76]. Therefore, besides anchoring cells, focal contacts play an important role in relaying signals from the material substrates to the cytoskeleton [77]. Consequently, formation of focal contacts are of enormous importance and potentially determine the success of the scaffolds [56,78].

For instance, Hunter *et al.* showed that cells forming the highest number of focal contacts exhibited a well spread and flattened morphology whereas those with the least number of focal contacts assumed a more rounded and less spread shape [78]. Also, it was reported that the shape of anchorage-dependent cells is the main regulator for the proliferation rate [78]. Flattened cells are firmly attached by well-defined attachment extensions and several lamellipodia, and have been demonstrated to have a higher rate of proliferation than cells assuming a rounded morphology, which are considered to be poorly attached [78-80].

Accordingly, since the degree of cell adhesion to a substrate can be reflected through the cell morphology and ultimately through the proliferation and phenotypic expression of

cells [56,76], these parameters may be related and can be used as an indicator of the affinity of the cells to a material [76,79].

3.7. SCAFFOLD REQUIREMENTS

The three main functions of bone tissue engineering scaffolds are: (1) providing temporarily a mechanical support, (2) guiding tissue regeneration and organization, (3) as well as promoting tissue formation and subsequent regeneration [23,38].

Several factors influence the patterns of cell adhesion and the biological responses to the different scaffolds. *In vitro*, the surface chemistry, microtopography and also the scaffold macroporosity and degradation behaviour affect the adsorption of biological molecules on the biomaterial and influence osteogenic cell attachment, viability, proliferation, and differentiation [19,81,82]. Hence, the design requirements such as scaffolding biomaterials and physical characteristics can significantly affect the whole development of engineered bone tissues [83]. Therefore, an ideal scaffold should generally have the following characteristics:

3.7.1. Chemistry

The surface chemistry is a main factor to cellular adhesion [84]. Cellular adhesions at the cell-material interface are mediated by proteins adsorbed from the surrounding medium onto the substratum [56,76]. The material ions modify the adsorption and orientation of proteins on the surface, and thus, also affect the subsequent cell binding [56]. Therefore, cells are sensitive to surface chemistry, such as material compositions, which may affects attachment and proliferation [56]. Osteoblasts can discriminate between subtle difference in chemistries and even between substrates having identical chemistry but little different crystallinity [85].

It is though that a poorly adhesive surface on which weak cell adhesion occurs, no traction and no net movement can be observed. However, a highly adhesive surface

allowing for a strong cell adhesion may also result in cell immobilisation [84]. An ideal surface chemistry should therefore allow for an intermediate adhesion force for an adequate cell attachment, that will promote proliferation and differentiation and also cell migration and scaffold colonization [23,84].

Accordingly, the bioactive potential of a scaffold is a key element. Bioactivity refers to the ability of a material to form a biologically active carbonated hydroxyapatite layer to which bone forms directly on the surface and chemically binds to it. This phenomena is referred to as bone bonding, and is due to an ion-exchange reaction between the bioactive materials and the surrounding body fluids, resulting in the precipitation of a hydrocarbonate apatite (calcium phosphate) layer equivalent to the mineral phase of bone [36,40,86]. The bone grows along these material's surfaces [18,87].

In addition, the scaffold material must be highly biocompatible to prevent an immunological reaction and must be bioresorbable [23]. The degradation by-products should be nontoxic and eliminated by the body without a detectable foreign body reaction [23,88].

A scaffold material having such adequate chemistry will promote cell attachment, proliferation, and differentiation [23].

3.7.2. Microtopography

Surface texture or microtopography plays an important role in the cellular response and adhesion [56,81,89]. It has been shown that osteoblast-like cells have a preference for rough surfaces and attach more to it [89]. Mesenchymal cells will detect subtle differences in surface roughness, and will accordingly adopt a variety of morphologies upon attachment [56]. In order for the cells to respond in such a way, the individual cell needs to be able to perceive the microtexture, e.g. approximately when peak heights are greater than 2 μm and the distance between the peaks are less than about 10 μm [90]. On smoother surfaces, the cells are able to spread, perhaps forming greater number of

hemidesmosomes as anchors to the substrate [56]. In contrast, on rougher surfaces, the cells appear to form focal contacts that allow the cells to span across the space between surface peaks [56,91,92]. Such modifications in attachment numbers and even distribution on a cell surface can result in new gene transcription, and new protein synthesis, ultimately affecting phenotypic expression [56]. For instance, it was previously reported that thin plasma-sprayed surfaces with coating of titanium, hydroxyapatite or tricalcium phosphate, enhanced bone formation around the implant due to surface chemistry, but also due to the surface that has acquired a rough topography and has influenced the numbers and types of focal contacts formation with their substrate [91,92]. Also, some experiments demonstrated that with increased surface roughness, osteoblastic cell differentiation was enhanced, with increased osteocalcin release [90].

3.7.3. Porosity, pore size

Regardless of the material from which they are formed, scaffolds must have the appropriate physical structure to serve as an osteoconductive matrix [18,34]. The microstructure of scaffolding material should be highly porous with interconnected pore network. In such scaffolds, the large void volume facilitates anchorage-dependent cell seeding, maximize attachment, migration and growth, extracellular matrix production, fluid circulation, and vascularization within the pore space throughout the scaffold structure [10,88,93].

The macroporosity of a scaffold is a critical factor in the determination of osteogenic cell migration and bone-matrix formation [19]. Although there are alternative views, previous researches tend to show that pore sizes ranging approximately between 300 to 400 μm would be optimal for several reasons including the following [94]:

First, since the type of tissue growing into the scaffold depends on the dimensions of the interconnecting pores, scaffold macroporosity is a crucial element for bone tissue engineering [18,19]. If pore sizes are less than 10-40 μm, fibrovascular tissue forms, and

if interconnections are from 40 to 100 μm, osteoid formation is encouraged. But in order to regenerate mineralized bone, pore sizes must be greater than 100 μm [18].

Second, it has been demonstrated that bone cells respond to gross morphology. For instance, osteoblasts have a preference for pore sizes ranging from 200-400 μm, stimulating migration, attachment and proliferation. It was though that these pore curvatures might provide optimal compression and tension on the cell's mechanoreceptors [56].

Third, cell bridging and occlusion by cells in the *in vitro* stage was reported to occur in both inorganic and organic scaffold materials with macropore of 200 μm or less [19]. Complete pore occlusions on the surface of a scaffold cause serious problems by preventing further cell and tissue ingrowth throughout the scaffold, essential once implanted into the patient, making the obtained polymer/cell constructs unsuitable as tissue engineering scaffolds [19]. Since it is solely dependant upon macroporosity, this can be avoided by increasing the nominal macropore size [19].

Fourth, bone ingrowth velocity has been reported to increase with pore size from 50 μm to 400-500 μm [88], and other *in vivo* studies demonstrated that 300 μm pores were suboptimal for bone ingrowth [95]. Therefore, pore size from 300-400 μm should fall in an optimal range for bone ingrowth.

3.7.4. Mechanical properties and degradation behaviour

The purposes of a scaffold result in conflicting scaffold design requirements and goals [38]. For instance, a dense scaffold would enhance mechanical properties whereas cell delivery would be promoted with highly porous structures [38]. Accordingly, in attempt to conciliate the physical and the mechanical requirements, scaffold porosity should be maximum as long as the mechanical features are not compromised [10,46]. Although this is a generally accepted concept, the optimal magnitudes of the mechanical requirements of a temporary scaffold are not established [38].

Nevertheless, compressive strength is though to be an important mechanical feature of porous scaffolds, especially for bone tissue engineering [38,96]. In order to allow adequate tissue development throughout the polymer/cell construct, the scaffolds should provide enough initial strength to preserve its structural integrity during long period of cell culture, until the seeded cells have formed a premature tissue that is mechanically strong enough to maintain itself [10,23,83,88,97,98].

Because the scaffold will start losing its mechanical strength as it degrades, its degradation and resorption kinetics should match tissue formation *in vitro* and/or *in vivo* to preserve its original physical and mechanical properties [10,23]. Such controlled degradation avoid collapse or stress shielding of the newly grown tissue, allowing the load-bearing activity to be transferred gradually from the scaffolding material to the newly developed bone tissue [10,99].

3.8. SCAFFOLD MATERIALS

Four categories of biodegradable biomaterials have been investigated by numerous groups, both *in vivo* and *in vitro* as candidates for bone tissue engineering scaffolds [36]: (i) synthetic organic materials: mainly the polyester family poly(α-hydroxyl acids) such as polylactide (PLA), polyglycolide (PGA), and their co-polymer (PLGA); (ii) synthetic inorganic materials: mainly hydroxyapatite, tricalcium phosphate, glass ceramics; (iii) organic materials of natural origin: collagen, alginate, agarose, hyaluronic acid derivatives, chitosan, and fibrin glue; and (iv) inorganic material of natural origin: coral and coralline hydroxyapatite [23,34,93,100].

3.8.1. Organic materials

Organic materials are widely used in several different applications, principally due to their availability in large variety of compositions and forms (solids, films, gels, etc.). They are also attractive because they can be fabricated readily into various shapes and structures with desired macroporous features [73,101].

All the polymers of natural and synthetic origins that have been investigated for use as scaffolds in tissue engineering are biodegradable, biocompatible and have their own distinctive characteristics [10,73,97]. However, both synthetic and natural biopolymers are mechanically weak, do not have good bioactivity, degrade too fast in physiological media loosing their mechanical properties and sterilization processes (autoclave, ethylene oxide, and Co irradiation) may alter polymer properties [97,101].

3.8.1.1. Polymer biomaterials

3.8.1.1.a. Synthetic polymers

Synthetic polymers can be produced in large quantities, and their synthesis allows for direct control over chemical composition [10,21]. Therefore, depending on the application, a desired set of physical specifications can be obtained when modifying the chemical structure with specific functional groups [10,21,73].

A vast majority of the research has focus on the synthetic polymers that already have the advantage of Food and Drug Administration approval as sutures [2,10]. These belong to the aliphatic polyester family poly(α-hydroxyl acids), mainly PLA, PGA, and their co-polymer PLGA. Another advantage of theses aliphatic polyesters resides in that, being extensively studied, their sterilizability and relative biocompatibility are well known [10]. They degrade by hydrolysis into non-toxic byproducts that can be metabolized and excreted [10]. Also, since PLLA is less crystalline than PGA, and more hydrophobic with its additional methyl group, variable rates of hydration and hydrolysis can be obtained with different combinations of PLA and PGA [10,93]. Various PLGA copolymers can be produced, with their corresponding physicomechanical properties [10,93].

Synthetic polymers, such as biodegradable polyesters, have succeeded to some degree in tissue engineering applications, but despite their attractive properties, they are limited by important drawbacks [97].

The general problems with these polymers are their low biocompatibility, the generation of acidic degradation by products, processing difficulties and loss that is too rapid of mechanical integrity while degrading [73-75,93,97]. Moreover, a key limitation with their use may be that they cannot behave biologically in a similar way from bone extracellular matrix (ECM), due to their hydrophobicity and lack of cell-recognition signals, affecting cells adherence and homogeneity of the distribution [102].

3.8.1.1.b. Natural polymers: chitosan biomaterials

The use of naturally occurring biomaterials is an alternate scaffold strategy offering distinct advantages [2,48]. These polymers may be much more similar to the native cellular milieu that has been optimized through evolution [2,48]. Scaffolding applications requires such natural materials, which are expected to have a higher biocompatibility and have demonstrated a better and faster healing process [103]. Natural polymers are promising in this field, however concerns about their availability in sufficient quantities has prompted the research on synthetic polymers as candidate materials [103].

Chitosan is a unique polysaccharide derived from partial de-acetylation of chitin, which is, after cellulose, the most abundant natural polysaccharide [104]. Found in arthropod exoskeletons, each year several million tons of chitin are harvested worldwide from the shell of shrimp, lobster, crab or krill [103,104].

With its chemical nature and biological properties, chitosan biomaterial is highly versatile [105]. The polymer has reactive amino and hydroxyl groups that provide many possibilities for covalent and ionic modifications. They can be easily modified with a large variety of groups that can be chosen to modify specific functionality such as biological and physical properties [100,106].

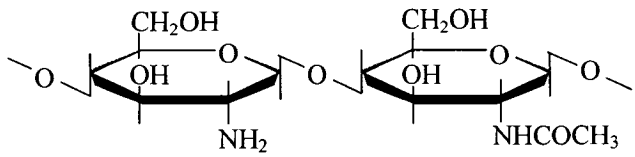

Figure 3.1. Molecular structure of chitosan. *The repeating units consist of β(1→ 4) linked D-glucosamine and of N-acetyl-glucosamine groups.*

Chitosan intrinsically possesses strong biological activity that has only been extensively studied in the past 20 years [105]. First, it is biocompatible, biodegradable, bioresorbable and has a hydrophilic surface, which facilitates cell adhesion, proliferation, and differentiation [43,88,107,108]. Second, with its cationic nature in physiological pH, chitosan mediates non-specific binding interactions with various proteins. Soluble proteins, most of which are negatively charge, may also be expected to have varying binding affinities to chitosan-based material [109]. Third, chitosan is made of glucosamine and *N*-acetyl-D-glucosamine units linked by one to four glycosidic bonds (Figure 3.1). The latter moiety is a structural molecule found in glycosaminoglycans [106,110], which is a polysaccharide occurring ubiquitously within ECM, including the one in bone and cartilage [106,109,111]. Glycosaminoglycans are know to be involved in several cell-cell/cell-matrix interactions, including specific bindings to growth factor receptors and adhesion proteins, and may thus modulates cell morphology, motility, differentiation, synthesis, and function [112].

With its hydrophilic and cationic nature, and its structure analogous to glycosaminoglycans, chitosan is expected to be endowed with related biological activity [108,109,112]. In fact, chitosan exhibits interactions with ECM components, immune cells and growth factors such as the fibroblasts growth factors found in trabecular bone tissue [113,114]. It also has mitogenic activity on several different types of cells including osteoblasts, and has been reported to contribute to the differentiation of osteoprogenitor cells and to enhance and facilitate bone formation [105,108,113,115]. These immunostimulatory and mitogenic activity may play a role in the integration of the biomaterial once implanted, by inducing local cell proliferation [109].

Chitosan has a number of other desirable properties for a tissue scaffold: it has anticoagulant properties, antibacterial and antifungal action [108,109]. Moreover chitosan has an excellent ability to be processed into porous structures for use in cell transplantation and tissue regeneration [109].

Although chitosan degradation is observed, the mechanism is not completely elucidated [108]. Chitosan is degraded mainly through hydrolysis mediated by lysozyme, which is a non-specific proteolytic enzyme found in various mammalian tissues [100]. Degradation byproducts are oligosaccharides of variable length [106]. The biodegradation rate appears to be inversely related to the degree of crystallinity, which usually vary with the degree of acetylation. *In vivo* degradation of highly deacetylated chitosan can take up to several months [109].

Chitosan properties have only been thoroughly studied in the last few decades [105], starting approximately when the scientific principles for use of the monomer N-acetylglucosamine in enhancing wound healing process were reported in 1960 [116]. In the 1970s, the role of chitosan in potentiating wound healing was documented for various animal models [117]. Since then, chitosan material has been widely investigated in a number of biomedical applications [103,104,117] from wound dressings [107,118,119], drug or gene delivery systems [120,121], and nerve regeneration [122] to space filling implant [123,124]. For tissue engineering, the utility of chitosan as a scaffolding material to support cell growth and proliferation has also been reported [88,97,106] involving many interesting interactions with cells ranging from osteoblasts [125,126], chondrocytes [106] and fibroblasts [118] to macrophages [119]. In many studies, positive cellular interactions were observed in the tissue repair and regeneration perspective [106].

As biomaterial, chitosan is an exceptional polysaccharide, the most promising of this class of materials [109]. It has excellent potential for engineering numerous tissue systems, including bone tissue, by serving as a structural base material on which normal tissue architecture is organized [100]. However, although pure chitosan has very

attractive properties, it lacks bioactivity and is mechanically weak [97]. These drawbacks limit its biomedical applications [102].

For these reasons, it is highly desirable to develop a hybrid material made of chitosan and an appropriate inorganic bioactive ceramics, hoping that it can combine the favourable properties of the materials, and further enhance tissue regenerative efficacy.

3.8.2 Inorganic materials

Inorganic bioceramics are known for their good biocompatibility, high compression resistance [101] and have also long been considered for a variety of medical applications [36]. Bioceramics with bioactive potential are of special interest as scaffolding materials to tissue engineers since they can form a continuous interface with surrounding bone tissue [36].

3.8.2.1. Calcium-based materials

The use of bioceramic and calcium salts is driven in part because of their similarity to the composition of bone mineral phase [18,20,26]. Bone inorganic salts fraction constitutes approximately 70% of its mass [18]. The major constituent of these salts is calcium phosphate in the form of hydroxyapatite but there are also substantially lower amounts of other calcium salts, such as calcium carbonate [18,21].

Since they are protein free, the inorganic salts components are biocompatible and nonimmunogenic, unlike the organic components of bone composed with cells, collagen and different macromolecules [18].

Moreover, biomaterials composed of these calcium salts, have been termed bioactive and osteoconductive [24,87]. Classification of some bioceramics in decreasing order of bioactivity level yields the following results: bioactive glass comes first, followed by tricalcium phosphate and finally hydroxyapatite, being less bioactive than the other

ceramics within that category. In addition, coral or more generally calcium carbonate crystals (aragonite and calcite), were found to show this important bonding property [40].

3.8.2.2. Natural coral, calcium carbonate biomaterials

As it was previously mentioned, numerous materials are currently used or studied either as substitutes for bone grafting or as scaffolding material for bone tissue engineering. They are mostly from synthetic origin, or have been synthetically modified to act properly as bone substitutes. However, natural coral is use as it is, in its entirely original nature. Natural coral has been used as bone substitutes because the exoskeleton edified some coral species have a three-dimensional structure, mechanical properties and a chemical composition similar to those of human bones [18,34,40,127-129]. Once harvested from the sea, the only treatments performed, before clinical use, are cleaning and sterilization [18,130].

Moreover, unlike many other biomaterials, about 30 years of extensive studies have largely recognized that natural coral can be an effective bone graft substitute when adequately applied [94]. Experimental studies started in 1975 and the first human clinical trials dates back to 1979 [131-133]. In 1987, Inoteb commercialized natural coral's bone graft substitutes under the trade name Biocoral® (Inoteb, Saint-Gonnery, France). Since 1980, natural coral has been widely used as a replacement biomaterial for bone grafts in different surgical specialties [130]: in orthopaedic surgery [134,135], cranial and maxillo-facial surgery [136-140], and neurosurgery [127,141]. Through these clinical conditions, calcium carbonate in the form of natural coral has been proven to be a successful bone substitute [142,143].

Natural corals, used as bone implants, are a hard form of corals known as stony corals, or scleractina (order madreporian), which composed in part the marine reefs. They are colonies of many little invertebrate animals named polyps, all grown originally from a single one. The polyps deposit an inorganic structure, which will be left behind as they divide and grow toward the surface, where there is nutrients and sunlight. The inorganic

skeleton formation process is reminiscent of the biological mineralization during bone or teeth formation [18,94].

Corals build a structure with a design characteristic to each species [18,94]. As a result, hundreds genera of stony corals built as many different exoskeletons with specific porosity and proportional mechanical properties [18]. Among them, only few genera have the required pore diameter and interconnectivity, two critical factors in the rate of coral resorption and bone regeneration, when use as bone implant [18,30,94]. Certain coral species, such as Porites and Goniopora, form an interconnecting porous network that mimics the matrix of either cancellous or cortical bone, and facilitates the ingrowth of fibrovascular and bone tissue into the coral implant [35,40,94,129,144,145]. Also, a limited number of other madreporic corals species have been employed for medical purposes, such as Acropora, Lobophyllia, Polyphyllia and Pocillopora [94].

Consequently, these coral species possess very important qualities for biomaterial submitted to bone substitution: they have inherent resorbability and osteoconductivity and adequate initial mechanical properties [40,144,146,147]. Moreover, they are characterized by their good biocompatibility *in vivo* [148] and *in vitro* [30,31,149], and have been proven to be bioactive [40,133].

In general, the degradation of calcium carbonate is mediated by physical and cellular mechanisms [94]. The dissolution process follows the principles of physical chemistry and varies with the local pH, the temperature, the solubility of the matrix as well as the surface area to volume ratio [18].The cellular process is believed to involve macrophages and osteoclasts [18,94]. The latter resorb the matrix in part through their carbonic anhydrase enzyme, producing carbon dioxide and hydrogen ions [18,94].

Most of the components found in bone are also found in coral. However, the ratio and composition of their organic and mineral components differ [94]. As mentioned previously, bone organic content is approximately 30% of its mass, while the coral

exoskeleton is constituted of only 1% of organic material (protein). Approximately 70% of bone weight is composed of calcium salts, including the main component, calcium phosphate in the crystal form of hydroxyapatite, and substantially lower amount of other calcium salts such as amorphous calcium phosphate mixed with calcium carbonate [18,21,94,150]. Coral's mineral composition is analogous to the calcium salts found in bones, and consists of over 97% calcium carbonate in the crystal form of aragonite [18,128]. Its crystal structure is similar to hydroxyapatite [128]. The remainder is constituted of a trace of different elements, which include magnesium, sodium, potassium, strontium, fluorine, and phosphorus (phosphate form) [40,94,151]. These oligoelements found in coral are known to have a significant effect in mineralization steps. Fluorine, known to induce osteoblasts proliferation, is found in coral at a concentration of 1.25 to 2.5 times higher than in bone. Strontium also helps bone mineralization and plays a protection role on calcification [94].

Research has largely demonstrated that coral scaffolds have all the principal qualities that an adequate bone graft substitute should possess, but like most biomaterials, it has no osteoinductive capacity and lacks osteogenicity [31,34,94,142,152]. However, adding growth factors and bone marrow cells can provide both missing properties [40,144]. In fact, observations *in vitro* and *in vivo* reported that the addition of either one of them enhance bone formation in comparison to natural coral exoskeleton alone, indicating that coral is a good carrier of growth factors [9,144,153] and supports osteogenic differentiation of transplanted marrow cells [30,33,49,148,152,154].

Petite *et al.* [34] have tissue-engineered artificial bone, using natural coral loaded with culture expanded marrow stromal cells assessed in a large segmental defect model in sheep. The scaffolds resorbed progressively until complete disappearance, leaving relatively mature remodelled bone, and in most favourable cases, lead to a complete recorticalization within 4 months. Thus, by combining MSCs and coral, large bone defects were reconstructed successfully.

Chen Gu's [49] experiments demonstrated that bone graft in the shape of a human mandibular condyle was restored successfully *in vivo* by using a combination of marrow-derived osteogenic cells seeded in natural coral scaffold.

New experimental and clinical studies continue to demonstrate that natural corals have qualities that are very important for biomaterial submitted to bone substitution which makes them excellent candidates as part of novel strategies for bone graft substitution and scaffolding material for bone tissue engineering.

3.9. COMPOSITE SCAFFOLD

In attempt to match the properties of natural trabecular bone, the development of bioactive materials with improved mechanical properties would be desirable. Although chemical modifications of many organic materials might provide interesting possibilities, composite materials which better mimic nature are the important future directions [73,155].

A composite consists of two or more chemically distinct phases separated by an interface [156]. As biomaterial, there are often two distinct parts: a discontinuous phase embedded within a continuous phase termed the matrix [156]. The former is usually the reinforcement phase, harder and stronger than the matrix, and is used either in particulates or fibre form to reinforce and stiffen the composite [36,157].

A composite is designed to combine all the favourable properties of each phase while minimizing the disadvantages of each when used alone [157]. More specifically, for bone repair applications, a composite is developed for three main reasons highlighted in the following [36,99]:

1. To enhance the mechanical properties of the scaffold.
2. To improved biological response to the construct.

3. To overcome the various effect of undesirable characteristics of each constituents when taken separately.

The properties of a composite are affected by a number of factors including [156,157]:

1. The specific characteristics of their constituent phases,
2. Their volume percentage,
3. Their distribution and
4. Their interaction with each other.

The variation of these factors allows an optimization of the overall performance of composites in a well-controlled manner [157].

Several types of biocomposites occur in nature. They are made from organic and inorganic phases, which play specific roles and combine to fulfill the mechanical and biological properties required in their particular functions [36]. For instance, skeleton, teeth or shells of organisms all possess such composite microstructure. In bone, organic ECM, composed partially of collagen, is associated with inorganic bone mineral, mainly hydroxyapatite. Similarly, in crab shell, chitin is combined with calcium carbonate [36,158,159].

Thus, the creation of biologically inspired combination of appropriate organic and inorganic materials diversifies the type of scaffolding matrix that can be developed and provide alternative choices to overcome many shortcomings of homogenous materials [36,101,155]. For this reason, many bioactive composite materials have been developed with the combination of bioceramic and biomedical polymers. The utilization of bioceramic's bioactivity, in the form of particulates reinforcing phase, enhance the composite's bioactive potential. Bioactive bioceramic-polymer composite systems can be expected to induce or enhance cell adhesion and the formation of adjacent tissue leading to a strong continuous interface while having better mechanical strength and stiffness than the polymer matrix alone [103,157].

3.9.1. Chitosan-bioceramic composites

Recently, many groups have proposed composites scaffolds of bioceramics particulates filler and biodegradable polymers including chitosan [43,97,102,125,160-162].

In a few studies, macroporous composite scaffolds made of a chitosan matrix reinforced by calcium phosphate powders, namely β-tricalcium phosphate (β–TCP) and calcium phosphate invert glass, were fabricated with a freeze-drying technique [102,160]. The results suggested that these composite scaffolds have good biocompatibility and that through the variation of the chitosan ratio to β–TCP and calcium phosphate invert glass, controllable pore structure, biodegradability, bioactivity, and improved compressive modulus and yield strength might be achieved [102].

Chitosan/tricalcium phosphate sponges were also developed and evaluated as scaffold for tissue engineered bone substitute by three-dimensional osteoblast culture [125]. Evaluations revealed that the composite sponges supported their proliferation and differentiation and suggest that these composite scaffolds are good candidate materials.

Various types of biodegradable chitosan-gel/β-TCP composite scaffolds were produced by freezing and lyophilization treatments. The study has demonstrated that the composite scaffolds have good biocompatibility and that their mechanical properties were significantly improved with the incorporation of β-TCP into chitosan-gel scaffolds [96].

Likewise, biodegradable hydroxyapatite/chitosan-gelatin composite scaffolds have been fabricated through a freeze-drying technique [161]. Changing the solid content and the compositional variables of the original mixtures allowed control of the porosities and densities of the three-dimensional scaffolds. Seeded with osteoblasts, observation indicated that the scaffold constructs is suitable for bone tissue engineering applications.

Furthermore, various other biodegradable polymers have been used as composite scaffolds including chitin matrixes [162] and other natural polymer such as collagen [163] as well as synthetic polyesters [75,164-166] showing similar improvements.

Freeze-dried hydroxyapatite-chitin matrixes composed with hydroxyapatite in 25, 50 and 75 wt% fractions were cultured *in vitro* with mesenchymal stem cells, which were induced into osteoblasts and then implanted *in vivo*. Interestingly, the study suggests that the hydroxyapatite particulate phase of the chitin matrix enhance the calcification and the degradation rate of the scaffolds [162].

The above-mentioned findings have shown that chitosan-based composites are promising candidates for bone repair and regeneration. Therefore, in our work, by taking advantage of these findings, we have proposed to use coral as inorganic component of the composite scaffold to develop macroporous composite biomatrices with improved physical and biological properties. Our hypotheses are based on two reasons:

1. The physical properties of the scaffold may be enhanced: By being incorporated into the chitosan polymers, coral is expected to act simultaneously as particulate reinforcing phase and gas-forming agent to result in a reinforced macroporous structure of large pore sizes. More specifically, a reaction between the coral's calcium carbonate and the acetic acid contained in the chitosan solution generates CO_2 that is expected to permeate through the chitosan and leads to the growth of large gas pores. Also, the toughness and plasticity of the chitosan phase could be combined to the strength of the coral phase and enhance its relatively weak mechanical properties [36,158].

2. The biological response to the construct may be improved: Since, *in vitro*, osteogenic cells are affected by the surface chemistry, microtopography, and scaffold macroporosity [19,81,167], the introduction of the coral particulate phase into the chitosan polymer may change these three parameters and influence favourably the cells behaviour. Moreover, the strong biological activity of chitosan [105] combined

with the excellent biocompatibility and the bioactive potential of corals, might promote bone bonding and allow for potentially better cells delivery and control over their differentiation [36,168].

3.10. CONCLUSION

Bone tissue engineering is a promising area offering the possible option of autogenous "living" bone grafting systems. MSCs-scaffold engineered construct can be expected to have sufficient new bone-forming capacity to regenerate skeletal tissues and to have a higher efficacy to heal large osseous defects [40,45].

But still, it is in early stages, and great challenges have to be overcome before the need for autograft may be reduced or eliminated [35,169]. The development of adequate biodegradable scaffolds is a main issue that needs to be resolved [157]. Although composite scaffolds investigated have improved properties, it seems that none of them have achieved the ultimate goal of bone tissue engineering: that is to create a device that has the capacity to replace autologous cancellous bone for the management of bony defect [75].

Further understanding of the natural tissues and advancement of the composite science are necessary in order to achieve this goal. Experimental and clinical studies are needed to test new candidate materials or composites having the required qualities for biomaterial submitted to bone substitution.

3.11. REFERENCES

1. Braddock M, Houston P, Campbell C, Ashcroft P. Born again bone: tissue engineering for bone repair. News Physiol Sci. 2001 Oct; 16: 208-13.
2. Rose FR, Oreffo RO. Bone tissue engineering: hope vs hype. Biochem Biophys Res Commun. 2002 Mar 22;292(1):1-7.

3. Finkemeier CG. Bone-grafting and bone-graft substitutes. J Bone Joint Surg Am. 2002 Mar; 84-A(3): 454-64.
4. Moore WR, Graves SE, Bain GI. Synthetic bone graft substitutes. ANZ J Surg. 2001 Jun; 71(6): 354-61.
5. Wright S. Commentary: The bone-graft market in Europe. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 591-596.
6. Parikh SN. Bone graft substitutes: past, present, future. J Postgrad Med 2002;48:142-8
7. Bucholz RW. Nonallograft osteoconductive bone graft substitutes. Clin Orthop. 2002 Feb; (395): 44-52.
8. Langer R, Vacanti JP. Tissue Engineering. Science. 1993 May 14;260(5110):920-6.
9. Ludwig SC, Kowalski JM, Boden SD. Osteoinductive bone graft substitutes. Eur Spine J. 2000 Feb;9 Suppl 1:S119-25.
10. Temenoff JS, Lu L, Mikos AG. Bone-tissue engineering using synthetic biodegradable polymer scaffolds. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp.454-459
11. Elsinger EC, Leal L. Coralline hydroxyapatite bone graft substitutes. J Foot Ankle Surg. 1996 Sep-Oct;35(5):396-9.
12. Kaufman E. Maxillary sinus elevation surgery: an overview. J Esthet Restor Dent. 2003;15(5):272-82; discussion 283.
13. Mendehal S. Commentary: The bone-graft market in the United States. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 585-590.
14. Cook SD, Baffes GC, Wolfe MW, Sampath TK, Rueger DC, Whitecloud TS 3rd. The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. J Bone Joint Surg Am. 1994 Jun; 76(6): 827-38.
15. Khan SN, Tomin E, Lane JM. Clinical applications of bone graft substitutes. Orthop Clin North Am. 2000 Jul;31(3):389-98.
16. Betz RR. Limitations of autograft and allograft: new synthetic solutions. Orthopedics. 2002 May;25(5 Suppl):s561-70.

17. Lucarelli E, Donati D, Cenacchi A, Fornasari PM. Bone reconstruction of large defects using bone marrow derived autologous stem cells. Transfus Apheresis Sci. 2004 Apr;30(2):169-74.
18. Shors EC. Coralline bone graft substitutes. Orthop Clin North Am. 1999 Oct;30(4):599-613.
19. Baksh D. Design strategies for 3-dimensional *in vitro* bone growth in tissue-engineering scaffolds. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 488-495.
20. Yaszemski MJ, Payne RG, Hayes WC, Langer R, Mikos AG. Evolution of bone transplantation: molecular, cellular and tissue strategies to engineer human bone. Biomaterials. 1996 Jan; 17(2): 175-85.
21. Laurencin CT, Ambrosio AM, Borden MD, Cooper JA Jr. Tissue engineering: orthopedic applications. Annu Rev Biomed Eng. 1999; 1: 19-46.
22. Damien CJ, Christel PS, Benedict JJ, Patat JL, Guillemin G. A composite of natural coral, collagen, bone protein and basic fibroblast growth factor tested in a rat subcutaneous model. Ann Chir Gynaecol Suppl. 1993;207:117-28.
23. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues-state of the art and future perspectives. J Biomater Sci Polym Ed. 2001;12(1):107-24.
24. Costantino PD, Hiltzik D, Govindaraj S, Moche J. Bone healing and bone substitutes. Facial Plast Surg. 2002 Feb;18(1):13-26.
25. Ladd AL, Pliam NB. Use of bone-graft substitutes in distal radius fractures. J Am Acad Orthop Surg. 1999 Sep-Oct;7(5):279-90.
26. Khan Y, Pratt LT, Laurencin CT. Bone Graft Substitute Materials. emedicine. 2004 mar 3.
27. Hollinger JO, Winn SR, Hu Y, Sipe R, Buck DC, Xi G. Assembling a bone-regeneration therapy. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp.435-439.
28. Delloye C, Cnockaert N, Cornu O. Bone substitutes in 2003: an overview. Acta Orthop Belg. 2003;69(1):1-8.

29. Ducheyne P, Qiu Q. Bioactive ceramics: the effect of surface reactivity on bone formation and bone cell function. Biomaterials. 1999 Dec;20(23-24):2287-303.
30. Doherty MJ, Schlag G, Schwarz N, Mollan RA, Nolan PC, Wilson DJ. Biocompatibility of xenogeneic bone, commercially available coral, a bioceramic and tissue sealant for human osteoblasts. Biomaterials. 1994 Jun;15(8):601-8.
31. Begley CT, Doherty MJ, Mollan RA, Wilson DJ. Comparative study of the osteoinductive properties of bioceramic, coral and processed bone graft substitutes. Biomaterials. 1995 Oct;16(15):1181-5.
32. Dalkyz M, Ozcan A, Yapar M, Gokay N, Yuncu M. Evaluation of the effects of different biomaterials on bone defects. Implant Dent. 2000;9(3):226-35.
33. Fricain JC, Bareille R, Ulysse F, Dupuy B, Amedee J. Evaluation of proliferation and protein expression of human bone marrow cells cultured on coral crystallized in the aragonite of calcite form. J Biomed Mater Res. 1998 Oct;42(1):96-102.
34. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. Nat Biotechnol. 2000 Sep;18(9):959-63.
35. Parikh SN. Bone graft substitutes in modern orthopedics. Orthopedics. 2002 Nov;25(11):1301-9; quiz 1310-1.
36. Laurencin CT, Lu H.H. Polymer-Ceramic Composites for Bone-Tissue Engineering. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 462-468.
37. Ishaug-Riley SL, Crane-Kruger GM, Yaszemski MJ, Mikos AG. Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. Biomaterials. 1998 Aug;19(15):1405-12.
38. Hollister SJ, Maddox RD, Taboas JM. Optimal design and fabrication of scaffolds to mimic tissue properties and satisfy biological constraints. Biomaterials. 2002 Oct;23(20):4095-103.
39. Yaszemski MJ, Oldham JB, Lu L, Currier BL. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 541-547.
40. Ohgushi H, Caplan AI. Stem cell technology and bioceramics: from cell to gene engineering. J Biomed Mater Res. 1999;48(6):913-27.

41. Gao J, Dennis JE, Solchaga LA, Awadallah AS, Goldberg VM, Caplan AI. Tissue-engineered fabrication of an osteochondral composite graft using rat bone marrow-derived mesenchymal stem cells. Tissue Eng. 2001 Aug;7(4):363-71.
42. Yoshikawa T, Ohgushi H, Akahane M, Tamai S, Ichijima K. Analysis of gene expression in osteogenic cultured marrow/hydroxyapatite construct implanted at ectopic sites: a comparison with the osteogenic ability of cancellous bone. J Biomed Mater Res. 1998 Sep 15;41(4):568-73.
43. Zhang Y, Ni M, Zhang M, Ratner B. Calcium phosphate-chitosan composite scaffolds for bone tissue engineering. Tissue Eng. 2003 Apr;9(2):337-45.
44. Ma PX, Choi JW. Biodegradable polymer scaffolds with well-defined interconnected spherical pore network. Tissue Eng. 2001 Feb;7(1):23-33.
45. Bruder SP, Jaiswal N, Ricalton NS, Mosca JD, Kraus KH, Kadiyala S. Mesenchymal stem cells in osteobiology and applied bone regeneration. Clin Orthop. 1998 Oct;(355 Suppl):S247-56.
46. Caplan AI. New logic for tissue engineering: Multifunctional and biosmart delivery vehicles. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 441-446.
47. Williams DF. Perspectives on the contributions of biomaterials and tissue engineering to bone repair, reconstruction, and regeneration. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 577-584.
48. Vacanti JP, Langer R, Upton J, Marler JJ. Transplantation of cells in matrices for tissue regeneration. Adv Drug Deliv Rev. 1998 Aug 3;33(1-2):165-182.
49. Chen F, Mao T, Tao K, Chen S, Ding G, Gu X. Bone graft in the shape of human mandibular condyle reconstruction via seeding marrow-derived osteoblasts into porous coral in a nude mice model. J Oral Maxillofac Surg. 2002 Oct;60(10):1155-9.
50. Long MW. Osteogenesis and bone-marrow-derived cells. Blood Cells Mol Dis. 2001 May-Jun;27(3):677-90.
51. Duguy N, Petite H, Arnaud E. Biomaterials and osseous regeneration. Ann Chir Plast Esthet. 2000 Jun;45(3):364-76.
52. Turksen K, Aubin JE. Positive and negative immunoselection for enrichment of two classes of osteoprogenitor cells. J Cell Biol. 1991 Jul;114(2):373-84.

53. Aubin JE. Osteogenic cell differentiation. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 21-28.
54. Sodek J, Cheifetz S. Molecular regulation of osteogenesis. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 31-42.
55. Aubin JE. Bone stem cells. J Cell Biochem Suppl. 1998;30-31:73-82.
56. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials. 1996 Jan;17(2):137-46.
57. Maniatopoulos C, Sodek J, Melcher AH. Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res. 1988 Nov;254(2):317-30.
58. Yoshikawa T, Noshi T, Mitsuno H, Hattori K, Ichijima K, Takakura Y. Bone and soft tissue regeneration by bone marrow mesenchymal cells. Mat Sci Eng C. 2001 17 :19-26.
59. Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. J Cell Sci. 1992 Jun;102 (Pt 2):341-51.
60. Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. Differentiation of human bone marrow osteogenic stromal cells *in vitro*: induction of the osteoblast phenotype by dexamethasone. Endocrinology. 1994 Jan;134(1):277-86.
61. Shalhoub V, Conlon D, Tassinari M, Quinn C, Partridge N, Stein GS, Lian JB. Glucocorticoids promote development of the osteoblast phenotype by selectively modulating expression of cell growth and differentiation associated genes. J Cell Biochem. 1992 Dec;50(4):425-40.
62. Leboy PS, Beresford JN, Devlin C, Owen ME. Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol. 1991 Mar;146(3):370-8.
63. Bellows CG, Aubin JE, Heersche JN. Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells *in vitro*. Endocrinology. 1987 Dec;121(6):1985-92.

64. Aubin JE. Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast differentiation. J Cell Biochem. 1999 Mar 1;72(3):396-410.
65. Kim, C. H., Cheng, S. L., & Kim, G. S. (1999). Effects of dexamethasone on activity, and cytokine secretion of normal human bone marrow stromal cells: mechanisms of glucocorticoid-induced bone loss. J Endocrinol, 162(3), 371-379.
66. Peter SJ, Liang CR, Kim DJ, Widmer MS, Mikos AG. Osteoblastic phenotype of rat marrow stromal cells cultured in the presence of dexamethasone, beta-glycerolphosphate, and L-ascorbic acid. J Cell Biochem. 1998 Oct 1;71(1):55-62.
67. Kii I, Amizuka N, Shimomura J, Saga Y, Kudo A.Cell-cell interaction mediated by cadherin-11 directly regulates the differentiation of mesenchymal cells into the cells of the osteo-lineage and the chondro-lineage. J Bone Miner Res. 2004 Nov;19(11):1840-9. Epub 2004 Aug 23.
68. Ferrari SL, Traianedes K, Thorne M, Lafage-Proust MH, Genever P, Cecchini MG, Behar V, Bisello A, Chorev M, Rosenblatt M, Suva LJ. A role for N-cadherin in the development of the differentiated osteoblastic phenotype. J Bone Miner Res. 2000 Feb;15(2):198-208.
69. Schiller PC, D'Ippolito G, Balkan W, Roos BA, Howard GA. Gap-junctional communication is required for the maturation process of osteoblastic cells in culture. Bone. 2001 Apr;28(4):362-9.
70. Cheng SL, Lecanda F, Davidson MK, Warlow PM, Zhang SF, Zhang L, Suzuki S, St John T, Civitelli R. Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation. J Bone Miner Res. 1998 Apr;13(4):633-44.
71. Goldstein AS. Effect of seeding osteoprogenitor cells as dense clusters on cell growth and differentiation. Tissue Eng. 2001 Dec;7(6):817-27.
72. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. J Cell Biochem. 1997 Feb;64(2):295-312.
73. Gunatillake PA, Adhikari R. Biodegradable synthetic polymers for tissue engineering. Eur Cell Mater. 2003 May 20;5:1-16.

74. Bergsma JE, de Bruijn WC, Rozema FR, Bos RR, Boering G. Late degradation tissue response to poly(L-lactide) bone plates and screws. Biomaterials. 1995 Jan;16(1):25-31.

75. Linhart W, Peters F, Lehmann W, Schwarz K, Schilling AF, Amling M, Rueger JM, Epple M. Biologically and chemically optimized composites of carbonated apatite and polyglycolide as bone substitution materials. J Biomed Mater Res. 2001 Feb;54(2):162-71.

76. Baxter LC, Frauchiger V, Textor M, ap Gwynn I, Richards RG. Fibroblast and osteoblast adhesion and morphology on calcium phosphate surfaces. Eur Cell Mater. 2002 Sep 30;4:1-17. Print 2002 Sep 30.

77. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular biology of the cell. Third edition. Garland Publishing, Inc, New York, 1994, pp.841.

78. Hunter A, Archer CW, Walker PS, Blunn GW. Attachment and proliferation of osteoblasts and fibroblasts on biomaterials for orthopaedic use. Biomaterials. 1995 Mar;16(4):287-95.

79. Rothamel D, Schwarz F, Sculean A, Herten M, Scherbaum W, Becker J. Biocompatibility of various collagen membranes in cultures of human PDL fibroblasts and human osteoblast-like cells. Clin Oral Implants Res. 2004 Aug;15(4):443-9.

80. Trylovich DJ, Cobb CM, Pippin DJ, Spencer P, Killoy WJ. The effects of the Nd:YAG laser on *in vitro* fibroblast attachment to endotoxin-treated root surfaces. J Periodontol. 1992 Jul;63(7):626-32.

81. Gomi K, Davies JE. Guided bone tissue elaboration by osteogenic cells *in vitro*. J Biomed Mater Res. 1993 Apr;27(4):429-31.

82. Sikavitsas VI, Temenoff JS, Mikos AG. Biomaterials and bone mechanotransduction. Biomaterials. 2001 Oct; 22(19): 2581-93.

83. Godbey WT, Atala A. *In vitro* systems for tissue engineering. Ann N Y Acad Sci. 2002 Jun; 961: 10-26.

84. Shieh M. Control of bone cell function on three-dimensional tissue engineering scaffolds. bug journal. 2000 ;3: 194-203.

85. Hambleton J, Schwartz Z, Khare A, Windeler SW, Luna M, Brooks BP, Dean DD, Boyan BD. Culture surfaces coated with various implant materials affect chondrocyte growth and metabolism. J Orthop Res. 1994 Jul;12(4):542-52.
86. Hench LL. Ceramics, Glasses, and Glass-Ceramics. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 73-83.
87. White E, Shors EC. Biomaterial aspects of Interpore-200 porous hydroxyapatite. Dent Clin North Am. 1986 Jan;30(1):49-67.
88. Wang JW, Hon MH. Sugar-mediated chitosan/poly(ethylene glycol)-beta-dicalcium pyrophosphate composite: mechanical and microstructural properties. J Biomed Mater Res. 2003 Feb 1;64A(2):262-72.
89. Bowers KT, Keller JC, Randolph BA, Wick DG, Michaels CM. Optimization of surface micromorphology for enhanced osteoblast responses *in vitro*. Int J Oral Maxillofac Implants. 1992 Fall;7(3):302-10.
90. Boyan BD, Schwartz Z. Modulation of osteogenesis via implant surface design. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 232-238.
91. Tisdel CL, Goldberg VM, Parr JA, Bensusan JS, Staikoff LS, Stevenson S. The influence of a hydroxyapatite and tricalcium-phosphate coating on bone growth into titanium fiber-metal implants. J Bone Joint Surg Am. 1994 Feb;76(2):159-71.
92. Burr DB, Mori S, Boyd RD, Sun TC, Blaha JD, Lane L, Parr J. Histomorphometric assessment of the mechanisms for rapid ingrowth of bone to HA/TCP coated implants. J Biomed Mater Res. 1993 May;27(5):645-53.
93. Hutmacher DW, Goh JC, Teoh SH. An introduction to biodegradable materials for tissue engineering applications. Ann Acad Med Singapore. 2001 Mar; 30(2): 183-91.
94. Demers C, Hamdy CR, Corsi K, Chellat F, Tabrizian M, Yahia L. Natural coral exoskeleton as a bone graft substitute: a review. Biomed Mater Eng. 2002;12(1):15-35.
95. Radder AM, Leenders H, van Blitterswijk CA. Application of porous PEO/PBT copolymers for bone replacement. J Biomed Mater Res. 1996 Mar;30(3):341-51.

96. Yin Y, Ye F, Cui J, Zhang F, Li X, Yao K. Preparation and characterization of macroporous chitosan-gelatin/beta-tricalcium phosphate composite scaffolds for bone tissue engineering. J Biomed Mater Res. 2003 Dec 1;67A(3):844-55.
97. Zhang Y, Zhang M. Three-dimensional macroporous calcium phosphate bioceramics with nested chitosan sponges for load-bearing bone implants. J Biomed Mater Res. 2002 Jul; 61(1): 1-8.
98. Zhang R, Ma PX. Porous poly(L-lactic acid)/apatite composites created by biomimetic process. J Biomed Mater Res. 1999 Jun 15; 45(4): 285-93.
99. Chaikof EL, Matthew H, Kohn J, Mikos AG, Prestwich GD, Yip CM. Biomaterials and scaffolds in reparative medicine. Ann N Y Acad Sci. 2002 Jun;961:96-105.
100. Madihally SV, Matthew HW. Porous chitosan scaffolds for tissue engineering. Biomaterials. 1999 Jun;20(12):1133-42.
101. Ramakrishna S, Mayer J, Wintermantel E, Leong KW. Biomedical application of polymer-composite materials: A review. Composites Science and Technology. 2001 Jul;61(9):1189-1224.
102. Zhang Y, Zhang M. Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. J Biomed Mater Res. 2001 Jun 5; 55(3): 304-12.
103. Khor E, Lim LY. Implantable applications of chitin and chitosan. Biomaterials. 2003 Jun;24(13):2339-49.
104. Hejazi R, Amiji M. Chitosan-based delivery systems: Physicochemical properties and pharmaceutical applications. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 213-237.
105. Klokkevold PR, Vandemark L, Kenney EB, Bernard GW. Osteogenesis enhanced by chitosan (poly-N-acetyl glucosaminoglycan) *in vitro*. J Periodontol. 1996 Nov;67(11): 1170-5.
106. Suh JK, Matthew HW. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials. 2000 Dec;21(24):2589-98.
107. Muzzarelli R, Biagini G, Pugnaloni A, Filippini O, Baldassarre V, Castaldini C, Rizzoli C. Reconstruction of parodontal tissue with chitosan. Biomaterials. 1989 Nov;10(9):598-603.

108. Domard A, Domard M. Chitosan: Structure-properties relationship and biomedical applications. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 187-212.
109. Matthew HW. Polymers for Tissue Engineering Scaffolds. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 170-186.
110. Tan W, Krishnaraj R, Desai TA. Evaluation of nanostructured composite collagen--chitosan matrices for tissue engineering. Tissue Eng. 2001 Apr;7(2):203-10.
111. Lahiji A, Sohrabi A, Hungerford DS, Frondoza CG. Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. J Biomed Mater Res. 2000 Sep 15;51(4):586-95.
112. Dumitriu S. Polysaccharides as biomaterials. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 1-45.
113. Cho BC, Park JW, Baik BS, Kwon IC, Kim IS. The role of hyaluronic acid, chitosan, and calcium sulfate and their combined effect on early bony consolidation in distraction osteogenesis of a canine model. J Craniofac Surg. 2002 Nov;13(6):783-93.
114. VandeVord PJ, Matthew HW, DeSilva SP, Mayton L, Wu B, Wooley PH. Evaluation of the biocompatibility of a chitosan scaffold in mice. J Biomed Mater Res. 2002 Mar 5;59(3):585-90.
115. Kind GM, Bines SD, Staren ED, Templeton AJ, Economou SG. Chitosan: evaluation of a new hemostatic agent. Curr Surg. 1990 Jan-Feb;47(1):37-9.
116. Reynolds BL. Wound healing III: Artificial maturation of arrested regenerate with an acetylated amino sugar. Am J Surg. 1960;26:113-117.
117. Prudden JF, Migel P, Hanson P, Friedrich L, Balassa L. The discovery of a potent pure chemical wound-healing accelerator. Am J Surg. 1970 May;119(5):560-4.
118. Ma J, Wang H, He B, Chen J. A preliminary *in vitro* study on the fabrication and tissue engineering applications of a novel chitosan bilayer material as a scaffold of human neofetal dermal fibroblasts. Biomaterials. 2001 Feb;22(4):331-6.
119. Ueno H, Mori T, Fujinaga T. Topical formulations and wound healing applications of chitosan. Adv Drug Deliv Rev. 2001 Nov 5;52(2):105-15.

120. Sato T, Ishii T, Okahata Y. *In vitro* gene delivery mediated by chitosan. effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. Biomaterials. 2001 Aug;22(15):2075-80.

121. Aiedeh K, Gianasi E, Orienti I, Zecchi V. Chitosan microcapsules as controlled release systems for insulin. J Microencapsul. 1997 Sep-Oct;14(5):567-76.

122. Yamaguchi I, Itoh S, Suzuki M, Osaka A, Tanaka J. The chitosan prepared from crab tendons: II. The chitosan/apatite composites and their application to nerve regeneration. Biomaterials. 2003 Aug;24(19):3285-92.

123. Muzzarelli RA, Biagini G, Bellardini M, Simonelli L, Castaldini C, Fratto G. Osteoconduction exerted by methylpyrrolidinone chitosan used in dental surgery. Biomaterials. 1993;14(1):39-43.

124. Muzzarelli R, Baldassarre V, Conti F, Ferrara P, Biagini G, Gazzanelli G, Vasi V. Biological activity of chitosan: ultrastructural study. Biomaterials. 1988 May;9(3):247-52.

125. Lee YM, Park YJ, Lee SJ, Ku Y, Han SB, Choi SM, Klokkevold PR, Chung CP. Tissue engineered bone formation using chitosan/tricalcium phosphate sponges. J Periodontol. 2000 Mar;71(3):410-7.

126. Lee JY, Nam SH, Im SY, Park YJ, Lee YM, Seol YJ, Chung CP, Lee SJ. Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials. J Control Release. 2002 Jan 17;78(1-3):187-97.

127. Roux FX, Brasnu D, Loty B, George B, Guillemin G. Madreporic coral: a new bone graft substitute for cranial surgery. J Neurosurg. 1988 Oct;69(4):510-3.

128. Lucas A, Gaudé J, Carel C, Michel J.-F, Cathelineau G. A synthetic aragonite-based ceramic as a bone graft substitute and substrate for antibiotics. International Journal of Inorganic Materials. 2001 Jan; 3(1): 87-94.

129. Vuola J, Bohling T, Kinnunen J, Hirvensalo E, Asko-Seljavaara S. Natural coral as bone-defect-filling material. J Biomed Mater Res. 2000 Jul;51(1):117-22.

130. Fricain JC, Alouf J, Bareille R, Rouais F, Rouvillain JL. Cytocompatibility study of organic matrix extracted from Caribbean coral porites astroides. Biomaterials. 2002 Feb;23(3):673-9.

131. Souyris F, Pellequer C, Payrot C, Servera C. Coral, a new biomedical material. Experimental and first clinical investigations on Madreporaria. J Maxillofac Surg. 1985 Apr;13(2):64-9.
132. Chiroff RT, White EW, Weber KN, Roy DM. Tissue ingrowth of Replamineform implants. J Biomed Mater Res. 1975 Jul;9(4):29-45.
133. Patat JL, Guillemin G. Natural coral used as a replacement biomaterial in bone grafts. Ann Chir Plast Esthet. 1989;34(3):221-5.
134. Patel A, Honnart F, Guillemin G, Patat JL. Use of madreporaria coral skeletal fragments in orthopedic and reconstructive surgery: experimental studies and human clinical application. Chirurgie. 1980;106(3):199-205.
135. Pouliquen JC, Noat M, Verneret C, Guillemin G, Patat JL. Coral substituted for bone grafting in posterior vertebral arthrodesis in children. Initial results. Rev Chir Orthop Reparatrice Appar Mot. 1989;75(6):360-9.
136. Yukna RA. Clinical evaluation of coralline calcium carbonate as a bone replacement graft material in human periodontal osseous defects. J Periodontol. 1994 Feb;65(2):177-85.
137. Yukna RA, Yukna CN. A 5-year follow-up of 16 patients treated with coralline calcium carbonate (BIOCORAL) bone replacement grafts in intrabony defects. J Clin Periodontol. 1998 Dec;25(12):1036-40.
138. Papacharalambous SK, Anastasoff KI. Natural coral skeleton used as onlay graft for contour augmentation of the face. A preliminary report. Int J Oral Maxillofac Surg. 1993 Oct;22(5):260-4.
139. Mora F, Etienne D, Ouhayoun JP. Treatment of interproximal angular defects by guided tissue regeneration: 1 year follow-up. J Oral Rehabil. 1996 Sep;23(9):599-606.
140. Marchac D, Sandor G. Use of coral granules in the craniofacial skeleton. J Craniofac Surg. 1994 Sep;5(4):213-7.
141. Roux FX, Brasnu D, Menard M, Devaux B, Nohra G, Loty B. Madreporic coral for cranial base reconstruction. 8 years experience. Acta Neurochir (Wien). 1995;133(3-4):201-5.

142. Gao TJ, Lindholm TS, Kommonen B, Ragni P, Paronzini A, Lindholm TC, Jalovaara P, Urist MR. The use of a coral composite implant containing bone morphogenetic protein to repair a segmental tibial defect in sheep. Int Orthop. 1997;21(3):194-200.
143. Soost F. Biocoral an alternative bone substitute. Chirurg. 1996 Nov;67(11):1193-6.
144. Demers CN, Tabrizian M, Petit A, Hamdy RC, Yahia L. Effect of experimental parameters on the *in vitro* release kinetics of transforming growth factor beta1 from coral particles. J Biomed Mater Res. 2002 Mar 5;59(3):403-10.
145. Vago R, Plotquin D, Bunin A, Sinelnikov I, Atar D, Itzhak D. Hard tissue remodeling using biofabricated coralline biomaterials. J Biochem Biophys Methods. 2002 Jan 4;50(2-3):253-9.
146. Fricain JC, Roudier M, Rouais F, Basse-Cathalinat B, Dupuy B. Influence of the structure of three corals on their resorption kinetics. J Periodontal Res. 1996 Oct;31(7):463-9.
147. Irigaray JL, Oudadesse H, Blondiaux G. Quantitative study of the coral transformations '*in vivo*' by several physical analytical methods. Biomaterials. 1990 Jul;11:73-4.
148. Roudier M, Bouchon C, Rouvillain JL, Amedee J, Bareille R, Rouais F, Fricain JC, Dupuy B, Kien P, Jeandot R, Basse-Cathalinat B. The resorption of bone-implanted corals varies with porosity but also with the host reaction. J Biomed Mater Res. 1995 Aug;29(8):909-15.
149. Guillemin G, Meunier A, Dallant P, Christel P, Pouliquen JC, Sedel L. Comparison of coral resorption and bone apposition with two natural corals of different porosities. J Biomed Mater Res. 1989 Jul;23(7):765-79.
150. LeGeros RZ. Calcium phosphates in oral biology and medicine. Monogr Oral Sci. 1991;15:1-201.
151. http://www.biocoral.com/
152. Vuola J, Bohling T, Goransson H, Puolakkainen P. Transforming growth factor beta released from natural coral implant enhances bone growth at calvarium of mature rat. J Biomed Mater Res. 2002 Jan;59(1):152-9.

153. Arnaud E, De Pollak C, Meunier A, Sedel L, Damien C, Petite H. Osteogenesis with coral is increased by BMP and BMC in a rat cranioplasty. Biomaterials. 1999 Oct;20(20):1909-18.

154. Ohgushi H, Okumura M, Yoshikawa T, Inoue K, Senpuku N, Tamai S, Shors EC. Bone formation process in porous calcium carbonate and hydroxyapatite. J Biomed Mater Res. 1992 Jul;26(7):885-95.

155. Prestwich GD, Matthew H. Hybrid, composite, and complex biomaterials. Ann N Y Acad Sci. 2002 Jun; 961: 106-8.

156. Alexander H. Composites. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 94-104.

157. Wang M. Developing bioactive composite materials for tissue replacement. Biomaterials. 2003 Jun;24(13):2133-51.

158. Muzzarelli C, Muzzarelli RA. Natural and artificial chitosan-inorganic composites. J Inorg Biochem. 2002 Nov 11;92(2):89-94.

159. Wang X, Ma J, Wang Y, He B. Structural characterization of phosphorylated chitosan and their applications as effective additives of calcium phosphate cements. Biomaterials. 2001 Aug;22(16):2247-55.

160. Zhang Y, Zhang M. Calcium phosphate/chitosan composite scaffolds for controlled *in vitro* antibiotic drug release. J Biomed Mater Res. 2002 Dec 5;62(3):378-86.

161. Zhao F, Yin Y, Lu WW, Leong JC, Zhang W, Zhang J, Zhang M, Yao K. Preparation and histological evaluation of biomimetic three-dimensional hydroxyapatite/chitosan-gelatin network composite scaffolds. Biomaterials. 2002 Aug;23(15):3227-34.

162. Ge Z, Baguenard S, Lim LY, Wee A, Khor E. Hydroxyapatite-chitin materials as potential tissue engineered bone substitutes. Biomaterials. 2004 Mar;25(6):1049-58.

163. Liu L, Zhang L, Ren B, Wang F, Zhang Q. Preparation and characterization of collagen-hydroxyapatite composite used for bone tissue engineering scaffold. Artif Cells Blood Substit Immobil Biotechnol. 2003 Nov;31(4):435-48.

164. Zhang K, Wang Y, Hillmyer MA, Francis LF. Processing and properties of porous poly(L-lactide)/bioactive glass composites. Biomaterials. 2004 Jun;25(13):2489-500.

165. Calandrelli L, Immirzi B, Malinconico M, Orsello G, Volpe MG, Della Ragione F, Zappia V, Oliva A. Biocompatibility studies on biodegradable polyester-based composites of human osteoblasts: a preliminary screening. J Biomed Mater Res. 2002 Mar 15;59(4):611-7.

166. Ma PX, Zhang R, Xiao G, Franceschi R. Engineering new bone tissue *in vitro* on highly porous poly(alpha-hydroxyl acids)/hydroxyapatite composite scaffolds. J Biomed Mater Res. 2001 Feb;54(2):284-93.

167. Wieland M, Sittig C, Brunette DM, Textor M, Spencer ND. Measurement and evaluation of the chemical composition and topography of titanium implant surfaces. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 488-495.

168. Taboas JM, Maddox RD, Krebsbach PH, Hollister SJ. Indirect solid free form fabrication of local and global porous, biomimetic and composite 3D polymer-ceramic scaffolds. Biomaterials. 2003 Jan;24(1):181-94.

169. Luyten FP, Dell'Accio F, De Bari C. Skeletal tissue engineering: opportunities and challenges. Best Pract Res Clin Rheumatol. 2001 Dec;15(5):759-69.

CHAPTER 4: Use of Natural Coralline Biomaterials as reinforcing and gas-forming agent for developing novel hybrid biomatrices: Microarchitectural and mechanical studies

Since most of mammalian cells are anchorage dependent [1,2], porous scaffolds for engineering tissue are needed to provide temporarily a mechanical support, promote cell attachment, growth, and guide tissue formation and organization in 3D [3,4].

The development of adequate biodegradable tissue engineering scaffolds is one of the main issues that need to be resolved [5]. The fabrication of new composite scaffolds with improved biological and physical properties is necessary. For these reasons, the fist objective of this thesis was to develop novel 3D macroporous chitosan-coral composites scaffolds hoping that it can combine the favourable properties of the materials and further enhance tissue regenerative efficacy. In addition to its excellent biological properties, the coralline particulate phase was judiciously chosen to act simultaneously as gas-forming and reinforcing agent. It was therefore hypothesized that, as particulate reinforcing phase, higher coral concentration would increase the mechanical strength of the chitosan-based scaffold, and that, as gas-forming agent, it would simultaneously favour large pore formation.

In order to ascertain this hypothesis, the second objective of this thesis was to study the coral:chitosan weight ratio parameter for its effects on the physical properties of the scaffolds. The relationship between scaffold microarchitecture and mechanical properties was assessed with a combination of SEM, compression tests, and micro-CT that recently has been used to assess the microarchitecture of macroporous tissue-engineering polymer scaffolds through the reconstruction of the three-dimensional structures and the quantification of material properties [6-9].

The scaffolds were also culture with MSCs to study the effect of the scaffold composition on cell behaviour through cell-material interaction and cellular morphology observation by SEM, which fulfilled the objective 3a of this thesis.

The current chapter presents the results obtained from the realisation of the objectives mentioned above, that have formed the subject of a paper that has been accepted for publication in the journal "Tissue Engineering" entitled:

"Use of Natural Coralline Biomaterials as reinforcing and foaming agent for developing novel hybrid biomatrices: Microarchitectural and mechanical studies", by Mylène Gravel, Razi Vago, Maryam Tabrizian.

REFERENCES

1. Risbud M. Tissue engineering: implications in the treatment of organ and tissue defects. Biogerontology. 2001;2(2):117-25.
2. Zhang R, Ma PX. Porous poly(L-lactic acid)/apatite composites created by biomimetic process. J Biomed Mater Res. 1999 Jun 15; 45(4): 285-93.
3. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues-state of the art and future perspectives. J Biomater Sci Polym Ed. 2001;12(1):107-24.
4. Hollister SJ, Maddox RD, Taboas JM. Optimal design and fabrication of scaffolds to mimic tissue properties and satisfy biological constraints. Biomaterials. 2002 Oct;23(20):4095-103.
5. Wang M. Developing bioactive composite materials for tissue replacement. Biomaterials. 2003 Jun;24(13):2133-51.
6. Taboas JM, Maddox RD, Krebsbach PH, Hollister SJ. Indirect solid free form fabrication of local and global porous, biomimetic and composite 3D polymer-ceramic scaffolds. Biomaterials. 2003 Jan;24(1):181-94.

7. Zeltinger J, Sherwood JK, Graham DA, Mueller R, Griffith LG. Effect of pore size and void fraction on cellular adhesion, proliferation, and matrix deposition. Tissue Eng. 2001 Oct;7(5):557-72.
8. Behravesh E, Timmer MD, Lemoine JJ, Liebschner MA, Mikos AG. Evaluation of the *in vitro* degradation of macroporous hydrogels using gravimetry, confined compression testing, and microcomputed tomography. Biomacromolecules. 2002 Nov-Dec;3(6):1263-70.
9. Cartmell S, Huynh K, Lin A, Nagaraja S, Guldberg R. Quantitative microcomputed tomography analysis of mineralization within three-dimensional scaffolds *in vitro*. J Biomed Mater Res. 2004 Apr 1;69A(1):97-104.

4. PAPER 1

Use of Natural Coralline Biomaterials as reinforcing and gas-forming agent for developing novel hybrid biomatrices: Microarchitectural and mechanical studies

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4.1. ABSTRACT

This paper introduces the first attempt of fabricating 3D macroporous composites of chitosan and natural coralline material having pore sizes of 300-400 μm exceeding the upper pore size limit of 250 μm obtained with freeze-dried chitosan-based scaffolds. Natural coral particulates sizing less than 20 μm, which is mainly composed of calcium carbonate ($CaCO_3$), was simultaneously used as reinforcing phase and gas-forming agent to obtain a structure with large pores and improved mechanical and biological properties. The reaction between the coralline material and the acidic chitosan polymer solvent, which produced carbon dioxide (CO_2), was rapidly stopped by the subsequent thermally induced phase separation step, leaving coralline particulates in the polymeric structure. Scaffolds containing five different proportions of coralline material (0, 25, 50, 75, and 100 wt%) were investigated. The coralline:chitosan weight ratio was studied for its effects on the physical properties of the scaffolds. The relation between scaffold

microarchitecture and mechanical properties was assessed with scanning electron microscope (SEM) along with microcomputed tomography (micro-CT) imaging and compression testing. The scaffolds were used in bone marrow cell culturing experiments to assess the effect of composition on cell behaviour through cell-material interaction and morphological observation by SEM. Higher coralline concentration increased the pore wall thickness and favoured large pore formation. Varying the coralline particulate to chitosan polymer ratio from 0 to 75 wt% increased the average pore size from 80 μm to 400 μm while the porosity decreased from 91% to 78%. The compressive modulus was improved proportionally with the coralline content, and the 75 wt% composites had a significantly higher modulus than other chitosan-based scaffold groups. More cells were observed on scaffolds with higher coralline content. The cell culture experiments indicated that the scaffolds containing coralline material might have a high cell affinity, since it allowed fast cell attachment and spreading.

Keywords: Bone engineering, chitosan-based scaffolds, natural coral particulates, reinforcing and gas-forming agent, microstructure, porosity, mechanical properties, mesenchymal cell-material attachment and spreading.

4.2. INTRODUCTION

The most common strategy for engineering bone tissue is to use a hybrid biomaterial, formed from a combination of culture-expanded osteogenic cells seeded onto an appropriate temporary scaffold, which mimics the natural extracellular matrix [1-4]. The scaffold then gradually degrades, and is eventually replaced by the newly formed tissues. Such scaffold must satisfy a number of demanding requirements, namely biocompatibility, porosity, mechanical properties and adequate biodegradation rate [5]. Since no single material currently available provides all these essential features that an ideal scaffold requires, a logical approach is to design a composite that combines the favourable properties of each component while minimizing their shortcomings when used as homogenous materials [6-9].

To obtain such a composite, many researchers have recently proposed composite scaffolds of bioceramic particulates, such as calcium phosphate in the form of hydroxyapatite or β–tricalcium phosphate, incorporated into biodegradable polymers including chitosan to fabricate macroporous composite scaffolds with reinforced matrices and improved bioactivity [3,10-14]. Although these composite scaffolds have improved properties, it seems that none of them have achieved the ultimate goal of bone tissue engineering to create a device that has the capacity to replace autologous cancellous bone for the management of bony defect [15].

In this work, chitosan composite scaffolds, reinforced with natural coralline material are proposed as scaffold for bone engineering. Chitosan is one of the most promising polysaccharides for engineering numerous tissue systems, including bone tissue [16,17]. Made of glucosamine and *N*-acetyl-D-glucosamine groups [18], a molecular structure analogous to glycosaminoglycans, and having a cationic nature in physiological pH, chitosan exhibits multiple biological activities [16,19,20]. It is biocompatible, biodegradable, bioresorbable and has a hydrophilic surface, which facilitates cell adhesion, proliferation, and differentiation [11,20,21,22]. It has mitogenic activity on several different types of cells including osteoblasts, and has been reported to contribute to the differentiation of osteoprogenitor cells and to enhance and facilitate bone formation [20,23-25]. Chitosan has the ability to be easily processed into porous structures [16]. The thermally induced phase separation (TIPS) technique is the most commonly mentioned approach to generate porous scaffold from chitosan [26]. This technique has also been called "freeze-drying" when the polymer solvent is an aqueous solution [27]. Porous structures can be easily produced with control over mean pore diameter by varying the freezing conditions and the chitosan concentration [17].

Although chitosan has many interesting properties, it lacks bioactivity and it is mechanically weak [14]. This is why many researches have undertaken to make chitosan-based composites. The addition of coralline derivatives is one of these approaches, since natural coralline has been widely used as a replacement biomaterial for bone grafts in

different bone defects [2,28]. It possesses very important qualities for bone substitution due to its resorbability, osteoconductivity, bioactivity [2,29], adequate initial mechanical properties [2,28,30,31] and its good biocompatibility [2,32-34].

Calcium carbonate ($CaCO_3$) is the inorganic constituent of the skeletal material of natural coral [28] which reacts easily with acids and generates CO_2. This chemical property of coral exoskeleton is explored in the present study. By incorporating coralline particles into the chitosan polymer, they are not only used as particulate reinforcing phase but also as gas-forming agent. Our hypothesis was that the incorporation of coralline powders into chitosan polymers will result in a macroporous composite scaffolds with (i) enhanced mechanical properties due in part to the toughness and plasticity of the chitosan phase combined with the strength of the coralline phase [6,35]; (ii) increased osteogenic cells attachment due to changes in surface roughness and chemistry, and (iii) improved macroporosity [36] due to the generation of CO_2 in the acidic solution used as solvent for chitosan polymer. It must be noted that the freeze-drying processes currently used for chitosan and chitosan-based materials can only produce pore sizes up to 250 μm [17,27,37].

Chitosan-based hydrogel scaffolds containing three different ratios of coralline particles (25, 50, and 75 wt%) were prepared. The coralline:chitosan weight ratio parameter was studied for its effects on the physical properties of the scaffolds compared with two other groups of scaffolds made of their corresponding homogeneous material, namely coralline material and chitosan. The relationship between scaffold microarchitecture and mechanical properties was assessed using SEM, micro-CT imaging, and compression tests. In order to study the effect of the scaffold composition on cell behaviour, the scaffolds were cultured with mesenchymal cells (MSCs) and cell attachment capabilities were studied by SEM.

4.3. MATERIALS AND METHODS

4.3.1. Scaffold preparation

Chitosan solution with a concentration of 2 wt% was prepared by dissolving high viscosity chitosan (WA 1186 cPs, MW range from ~300–800 kDa, deacetylation degree 84.9 from Vanson HaloSource,) in a 0.2 M acetic acid. The solution was stirred at room temperature for 8 h and filtered through a fine cloth.

Coral skeletal material was obtained using small coral cores, from the species *Porites lutea,* harvested at the Seychelles archipelago. Exoskeleton blocs had an average pore size of 150 μm and an open porosity between 47-51%. They were soaked in NaOH 2N for 5 min, then treated with H_2O_2 30% for 10 min to remove trapped particles, debris and organic remnants. They were then broken in small pieces and shaped with sand paper to obtain flat squared coralline scaffolds measuring approximately 2x5x5 mm^3. The prepared coralline scaffolds were rinsed with distilled water and sterilized with an autoclave at 121°C for 40 min and oven-dried overnight at 80°C.

For chitosan-based scaffold preparation (Figure 4.1), the coralline particulate was obtained by milling solid coralline material for 8h in a rotating micromill. The particles were subsequently sieved through 20 μm U.S. standard sieve. Different proportion of coralline particulates (25, 50, and 75 wt%), sizing less than 20 μm, were added into the chitosan solution. The mixture was continuously stirred for 10 minutes to obtain homogeneous slurry. The reaction between the coralline and the acidic chitosan solvent produced CO_2, and led to the formation of a viscous bubbly solution. Chitosan as reference materials and the chitosan composite mixtures were then poured into 96 multi-well polystyrene culture dishes. Samples were rapidly transferred into a freezer at –80°C overnight to solidify the solvent, stop the gas-forming reaction and induce a solid-liquid phase separation. The samples were then freeze-dried for 4 days at a temperature of –52°C.

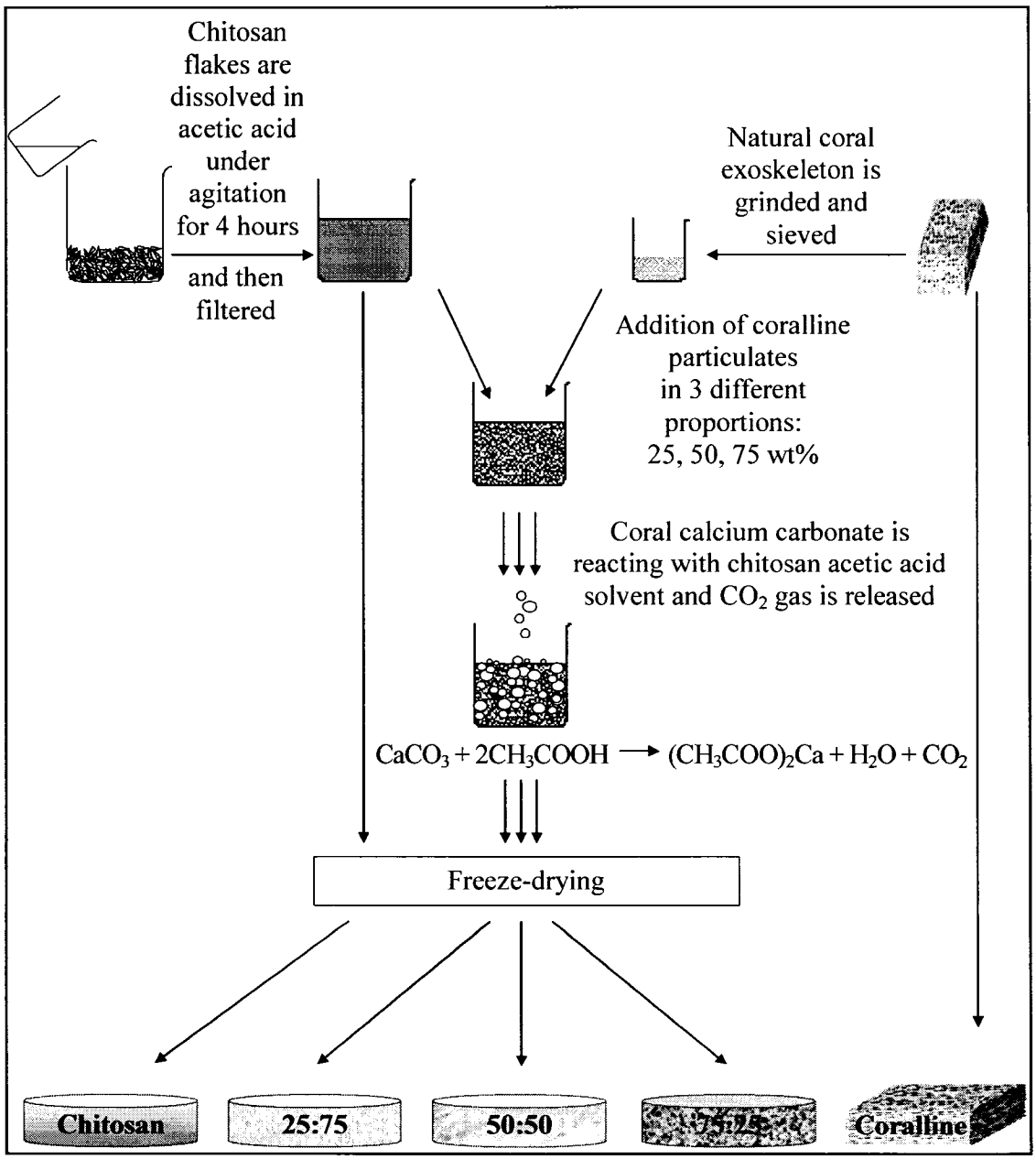

Figure 4.1. Schematic representation describing the production of the 5 groups of scaffolds. *The coralline particles, introduced in 3 different ratios into chitosan scaffolds, act both as a gas-forming and particulate-reinforcing agent: The reaction between the coral biomaterial and the acidic chitosan polymer solvent produced CO_2 which permeated through the chitosan to form pores. The reaction was rapidly stopped by freeze drying, leaving coral particulates reinforcing the polymeric porous structure.*

After lyophilisation, samples were neutralized by immersing them in absolute ethanol for 1h, followed by ethanol 70%/water (v/v) and 50% for 30 min each. The pH was adjusted with phosphate buffered saline (PBS) to 7.4 and scaffolds were rinsed with double-distilled water. The hydrated samples were frozen at –80°C, and cut with a razor blade into disks measuring approximately 2.5 mm in height and 5.5 mm in diameter. Finally, the scaffolds were freeze-dried and kept in a closed environment until use.

4.3.2. Microarchitectural analysis

The morphology of the scaffolds was examined with SEM (Hitachi field emission s-4700). The pore sizes were estimated from the SEM micrographs. Prior to SEM analysis, the samples were sputter-coated with gold-palladium under an argon atmosphere.

A Micro-CT system (SkyScan 1072, Belgium) was used to obtain 3D images from which samples' microarchitectural parameters such as porosity has been determined. The samples (n=4 for each coralline ratio group) were scanned using an energy of 39 kV and a current of 244 μA (9.5 watt power). A 180 degree scan was performed using a stepwise rotating angle of 0.45 degree with an integration time of 1782 msec for each image acquired with a solution of 4.56 μm. The cross-sections were reconstructed using Cone-Beam Reconstruction Software (SkyScan), having a distance 9.12 μm between each cross-section. Reconstructed array was shown as a half-tone image of cross section with linear conversion to 256-grades of grey inside selected density intervals which was kept the same for a sample group. Samples were analyzed with 3D Realistic Visualization software (Skyscan) to quantify the scaffolds architecture. The size of the region of interest and the threshold value was the same for all the samples in order to be comparable and to cover their maximum volume. To distinguish pore from material, the segmentation method was used. The method consisted of selecting a threshold value in the 256-gray scale where each voxel is considered either material or pore.

4.3.3. Compressive mechanical properties

The compressive equilibrium modulus was assessed from a stress relaxation function, using a Mach-1™ A400.25 mechanical tester (Biosyntech, Laval, PQ) reported to be

accurate for the measurement of the mechanical properties of both biomaterials and biological tissues [38-44]. During the tests, the scaffolds were kept immersed in phosphate buffered saline solution (PBS). Using a load cell of 150g, the scaffolds were slowly subjected to 5 sequential step compressions of 15, 30, 45, 60%, 75% of strain, in uniaxial unconfined compression configuration. Four scaffolds per group were used. Under compression, the swollen scaffolds were thus allowed to expand in the radial direction and to expel the liquid through their porous media. Consequently, for each compression step, the load rapidly increased to the maximal strain and then relaxed to its equilibrium value (equilibrium stiffness). Equilibrium was defined as a change in the slope of the load relaxation curve less than 0.2 g/min, i.e. stabilized slope close to zero. A fixed relaxation time of 240 sec was used at each compression step to reach this equilibrium. The equilibrium modulus for each step was then determined from the ratio of the equilibrium stress minus the initial stress over the strain [45].

4.3.4. Cell culture

MSCs (mouse cell line CRL-12424 from ATCC, Manassa, VA, USA), were cultured for 5 passages in Dulbecco's modified Eagle medium (DMEM) medium (Gibco, Burlington, ON, Canada), supplemented with 4 mM-glutamine, 4.5 g/L glucose, and 25 mM HEPES buffer, 10% fetal bovine serum (FBS) (Gibco), 10 U/ml penicillin G sodium, 10 μg/ml streptomycin and 25 μg/ml amphotericin B as Fungizone (Gibco), and 100 μg/ml L-ascorbic acid (Sigma-Aldrich, Oakville, ON, Canada).

A low-density cell seeding was assessed in order to avoid cell-cell contact and to achieve easier visualization of individual cell interaction with the scaffold material. In a 48 multi-wells plate, aliquots of 10 μl and 40 μl cell suspension were seeded onto the dry coralline and chitosan-based samples placed in wells, resulting in a seeding density of $5x10^5$ cells/cm^2. 150 μl of medium were added into each well. The scaffolds were incubated for 12 h to allow cells to attach to surface, and were then transferred into 35 mm tissue culture polystyrene dishes. Media were changed every 2-3 days. The cell-material interactions were studied after 3, 7, 21 days with SEM (Hitachi field emission s-4700). Coralline and chitosan scaffolds were used as reference materials.

4.3.5. Cell morphology analysis by SEM

The morphology of cells was examined with SEM. The cell-seeded scaffolds were rinsed twice with PBS, fixed with 2.5% glutaraldehyde in 0.14M sodium cacodylate (pH 7.4) for 24h at 5°C and dehydrated by 30, 50, 70, 80, 90, and 100% ethanol for 10 min for each. Further substitution to amyl acetate was performed through four graded bath of amyl acetate: ethanol (25:75), followed by (50:50), (75:25), and (100%). Prior to SEM observation, samples were critically point dried and covered with a thin layer of gold-palladium through sputtering under an argon atmosphere.

4.3.6. Statistical analysis

Multiple samples were collected for porosity measurements as well as for mechanical characterization. Data were reported as means ± standard deviations. One-way analysis of variance (ANOVA) with Scheffe's multiple comparison tests was used to assess the statistical significance between the porosity of the groups. The same ANOVA statistical analysis using Tukey's multiple comparison tests was performed to evaluate the difference within each compression step.

4.4. RESULTS

4.4.1. Analysis of compressive mechanical properties

The measurement of compressive equilibrium modulus showed that the chitosan-based scaffolds were reinforced proportionally by the addition of coralline powder phase (Figure 4.2). Although this trend was not supported by a significant difference for the 0, 25, and 50 % coralline weight ratios, the 75 wt% composites modulus was significantly higher ($p<0.001$) than all the other scaffold groups as soon as 30% strain was applied.

4.4.2. Analysis of scaffold morphology

The presence of the coralline particles in the polymeric scaffolds considerably changed the surface morphology (Figure 4.3 and 4.4). The concentration of rough coral particles observed on the composite scaffold walls and on the surfaces increased with the coralline

ratio. The distribution of coralline particles was homogeneous and no trend to agglomeration has been observed.

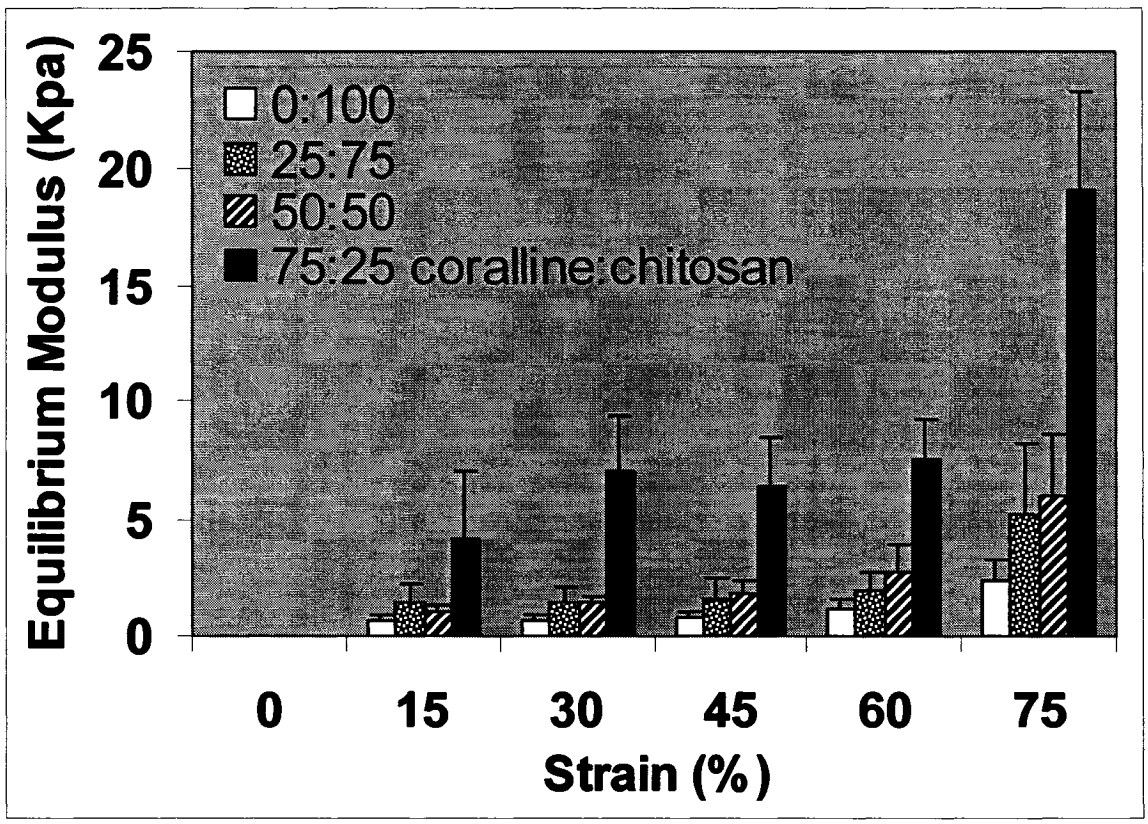

Figure 4.2. The compressive equilibrium modulus of macroporous 0:100, 25:75, 50:50, and 75:25 coralline:chitosan composites. *Equilibrium mechanical testing was conducted between 0% and 75% strain in 5 steps of 15% strain increments. Error bars represent means standard deviations for n = 4.*

As indicated in Figure 4.5, an increase of coralline content slightly and linearly decreased porosities allowing good control over the porosity of the chitosan-based scaffold. Significant differences were found only between the 0%-50 w% and between the 25-75 w% groups with $p<0.1$. Increasing the coralline content from 0% to 75 wt% decreased significantly ($p<0.01$) the porosity from 91% (± 0.44) to 77.85% (± 6.96).

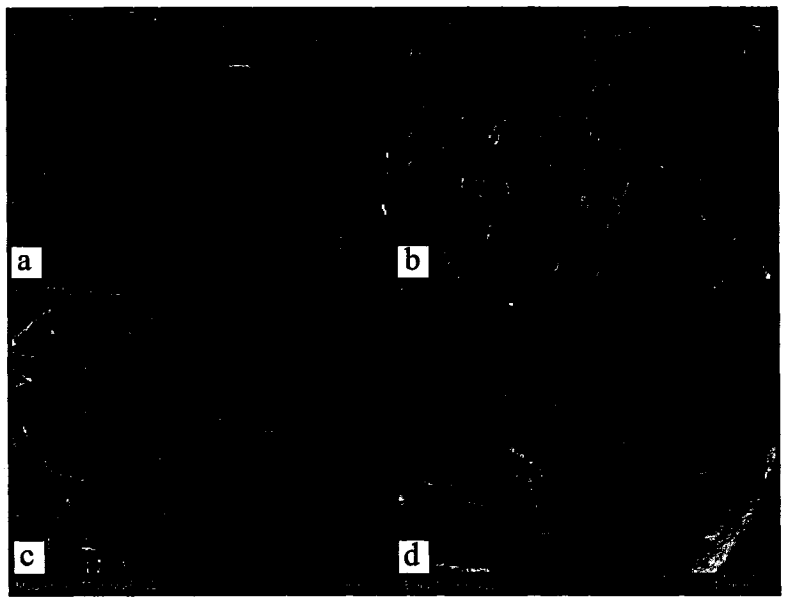

Figure 4.3. Cross-sectional SEM micrographs of chitosan-based scaffolds containing varying amount of coralline material. *Representation (x50) of* ***a.*** *0:100;* ***b.*** *25:75;* ***c.*** *50:50;* ***d.*** *75:25 coralline:chitosan weight ratios. The upper insets illustrate an x500 high magnification of corresponding scaffold.*

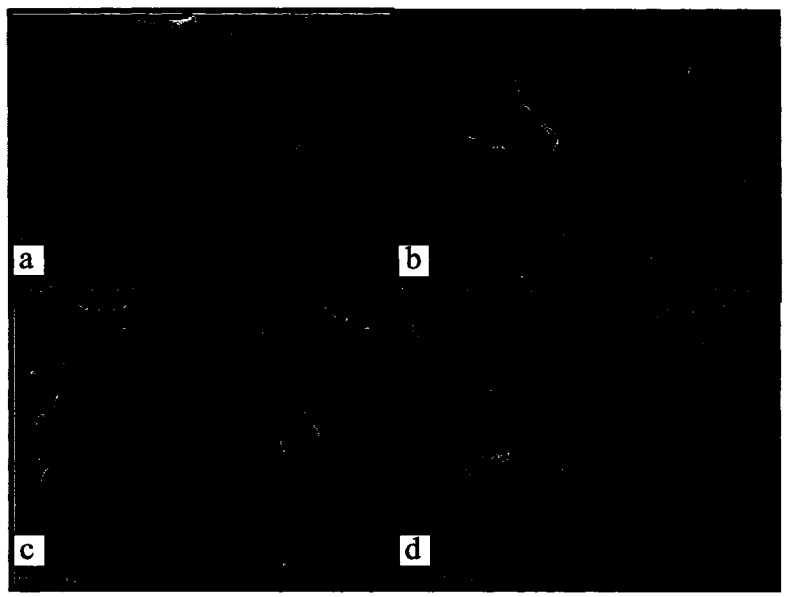

Figure 4.4. SEM micrographs of chitosan-based scaffold surfaces containing varying amount of coralline material. *Representation (x5k) of* ***a.*** *0:100* ***b.*** *25:75,* ***c.*** *50:50, and* ***d.*** *75:25 coralline:chitosan weight ratios.*

In addition to above-mentioned structural characteristics, coralline biomaterial concomitantly created larger pores into composite scaffolds. The evolving gas permeated through the chitosan and led to the growth of pores. The pore size as revealed by SEM images clearly increased with coralline content from 80 μm (average size for the chitosan scaffold) up to a mean of approximately 400 μm for the 75:25 coralline:chitosan wt% composite scaffolds, sometimes reaching a maximum pore sizes of about 1000 μm (Figures 4.3 and 4.6). The more coralline content there was, the more evolving gas permeated through the chitosan, leading to the growth of larger gas pores. The coralline:chitosan composites resulted in an open pore macrostructure on the surfaces, as demonstrated in both SEM micrographs (Figure 4.3) and the micro-CT reconstruction image (Figure 4.6).

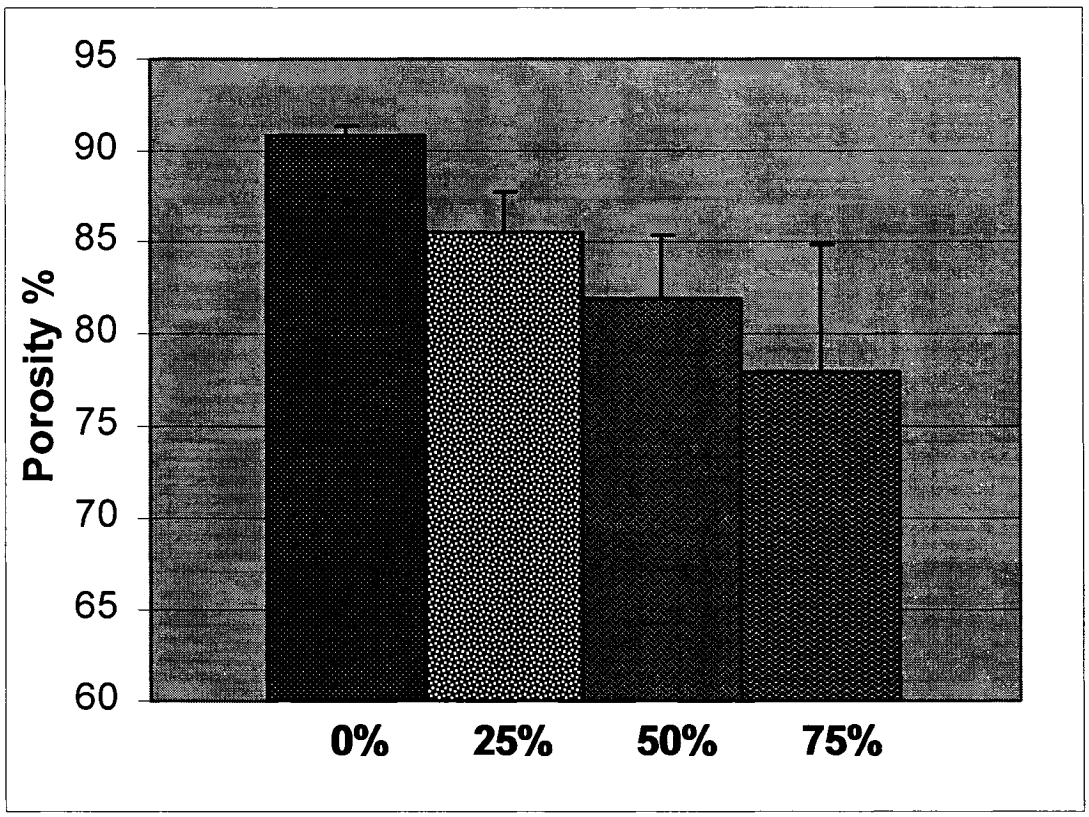

Figure 4.5. Micro-CT analyses showing different porosities for the chitosan-based scaffolds containing 0, 25, 50, and 75 wt% of coralline material. *Error bars represent means ± standard deviations for n = 6. (n = 4 for 0% study group) (0%-50% p<0.1, 25%-75% p<0.1, 0%-75% p<0.01).*

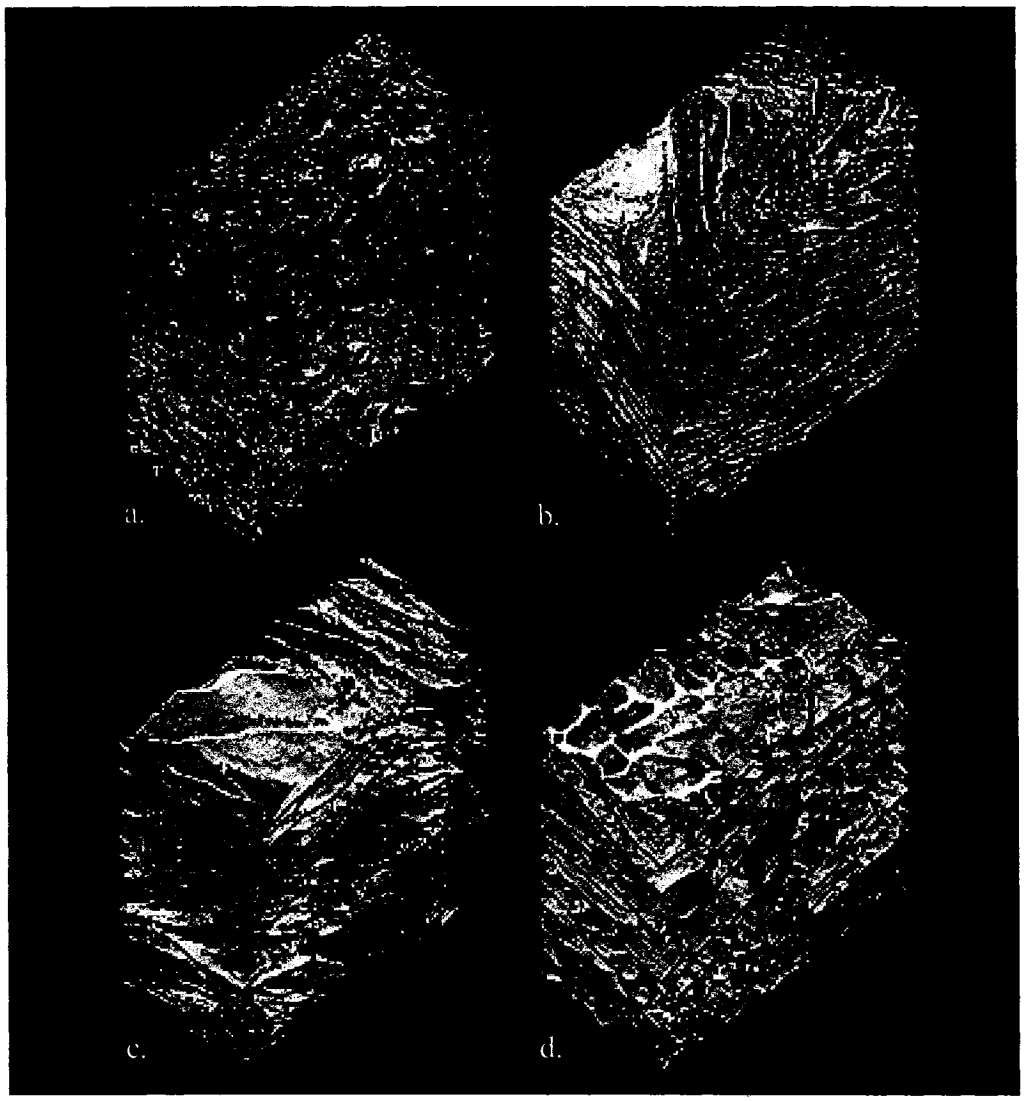

Figure 4.6. Representative micro-CT images of the macroporous scaffolds. ***a.*** *0:100;* ***b.*** *25:75;* ***c.*** *50:50;* ***d.*** *75:25 coralline:chitosan composites.*

The pore uniformity of the composite microstructures was affected by the presence of coralline material. The pure chitosan scaffolds had a homogeneous three-dimensional porous structure with small pore size range regular in shape. While adding 25 wt% of coral particulates did not seem to affect significantly the uniformity of the pore morphology, adding 50 or 75 wt% of coralline material into chitosan scaffolds resulted in structures with irregular pore morphology and larger pore size.

4.4.3. SEM analysis of cell attachment and spreading onto and into scaffold

The relationship between the scaffolds properties and their biological performance was investigated through cell adhesion and morphological studies. Throughout the three weeks of cell culture, the cells grown on coralline surfaces assumed distinct morphology compared to the cells grown on the chitosan-based scaffolds. During the entire study, every single MSC grown on the coralline scaffolds systematically exhibited highly flattened star shape morphology with many projections. As soon as one week after the seeding, a great number of cells were observed on those scaffolds starting to form a thin cell layer on the surface (Figure 4.7a). In contrast, the great majority of cells grown on the chitosan scaffolds, remained rounded for more than one week. Figure 4.7b shows such typical round cells found on chitosan scaffolds for which the cell spreading was considerably delayed. After one week of culture, few cells randomly distributed could be observed. Although most of cells were also round after three days of culture on the coralline:chitosan composite scaffolds, some spread cells having large and extended cell bodies with signs of activity such as microvilli, fibre-like processes and pseudopods were also found (Figure 4.7c).

After three weeks of culture, the cells covering the surfaces were quite different among the scaffold groups and seemed to be influenced by the coralline ratio (Figure 4.8). On the coralline scaffolds, a thick layer of cell had been laid down over the entire porous network and caused complete pore occlusions (Figure 4.8a).

On the 50:50 and 75:25 wt% coralline:chitosan scaffolds, a cell layer was covering the surfaces without blocking the lager pores (Figure 4.8c). Interestingly, virtually all of spread cells were forming pseudopods with coralline particles. The chitosan scaffolds were still characterized by the presence of mostly isolated cells along with few cell aggregations into the scaffold even though some signs of cell spreading and microvilli and fibre-like processes could be observed (Figure 4.8b).

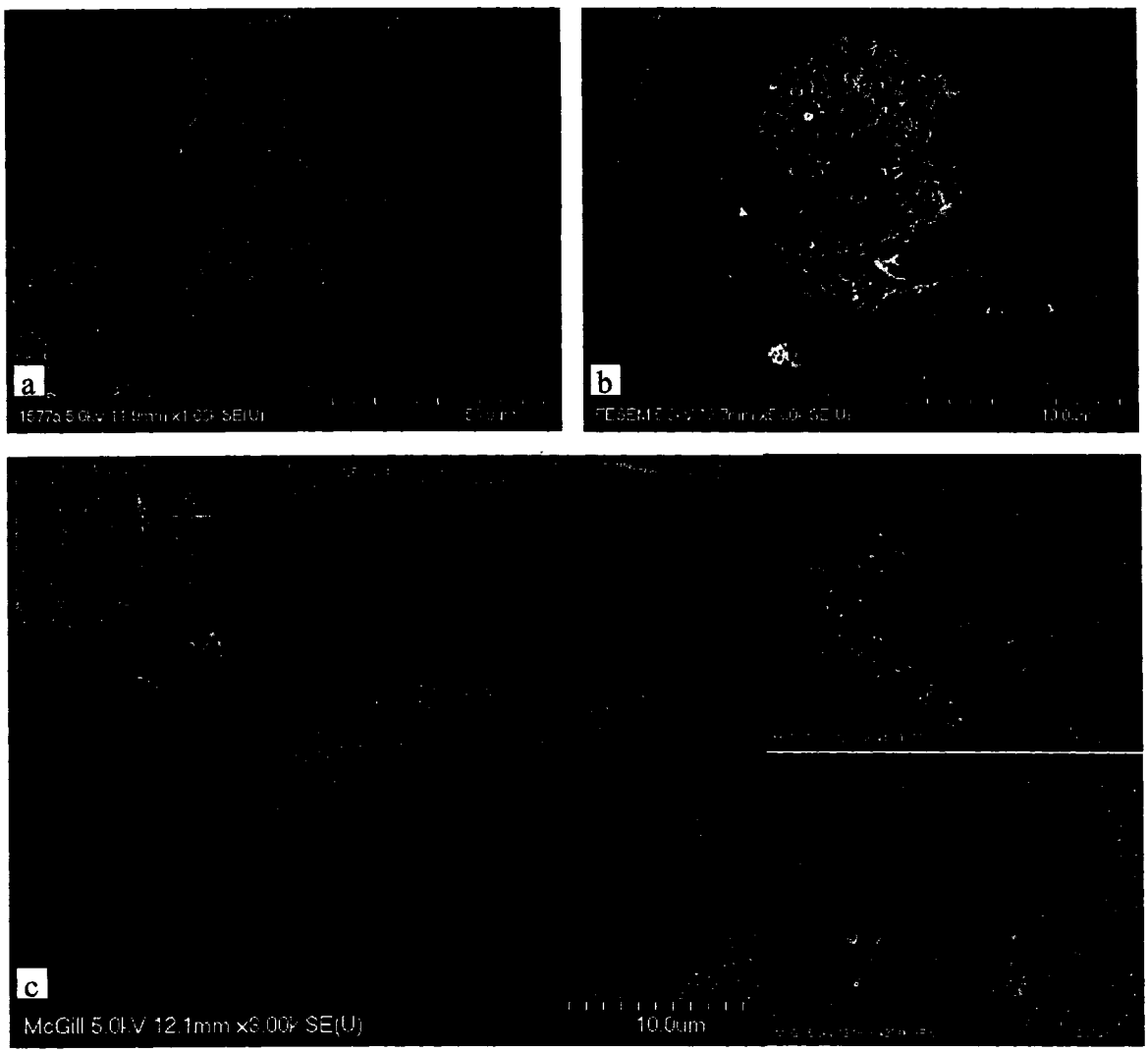

Figure 4.7. SEM micrographs showing distinct differences in morphology between cells cultured for three days on coralline and chitosan-based scaffolds. ***a.*** *Cells on coralline scaffolds exhibiting flattened star shape morphology with numerous microspikes and filopodia, sometimes 100 μm long (magnification x1k, inset x20k);* ***b.*** *Cells on chitosan surface exhibiting a rounded shape (magnification x5k);* ***c.*** *Spread cells on 25:75 coralline:chitosan composite scaffolds developing pseudopods with coral particles, representative the common feature of cell-coralline:chitosan scaffold interactions (magnification x3k, the upper and lower insets are a 10k and 20k high-magnification of filopodia).*

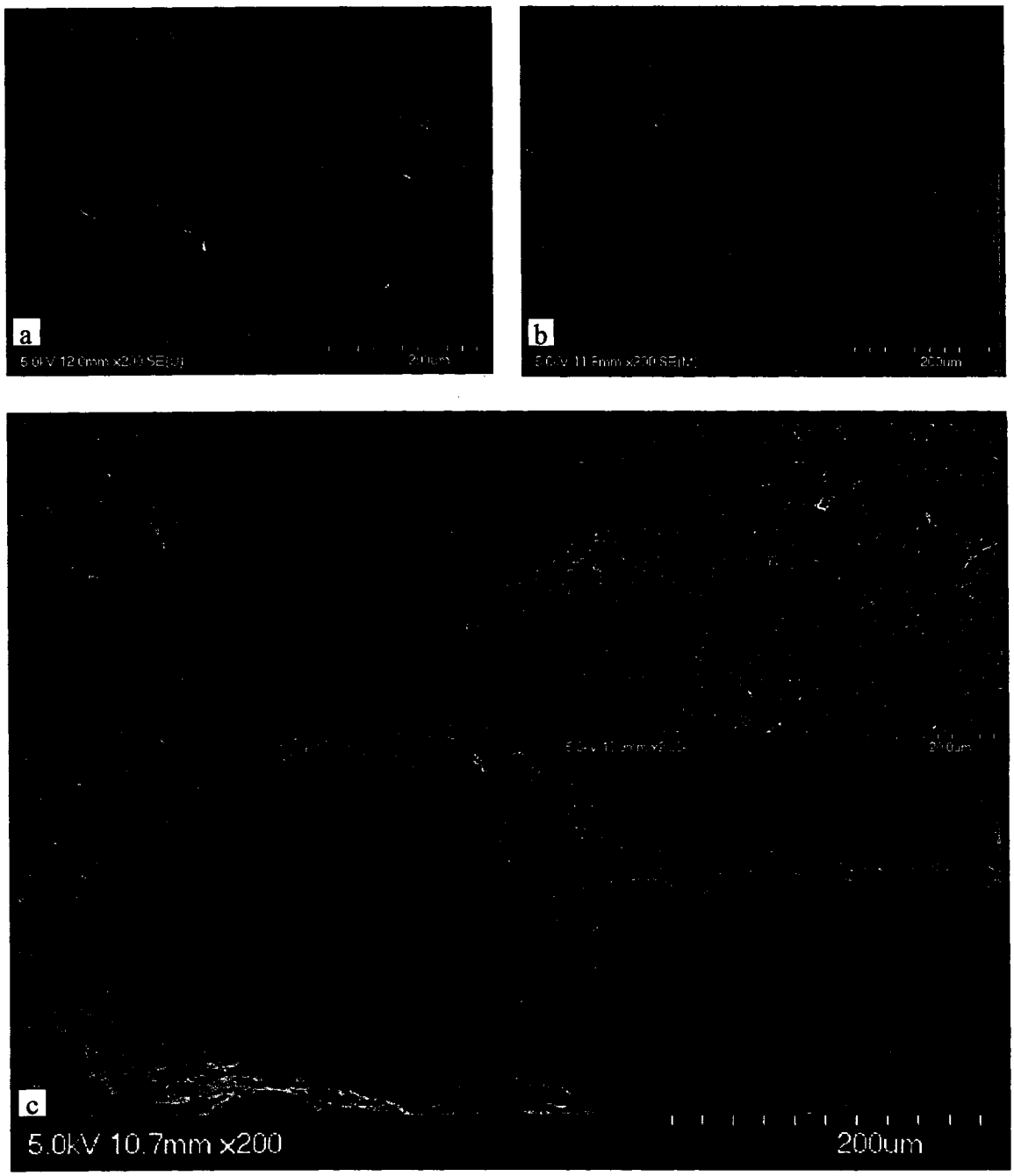

Figure 4.8. SEM micrographs of samples cultured with mice mesenchymal stem cells at 3 weeks. *(Magnification x200, insets x2k).* ***a.*** *Cell sheet on the coralline scaffolds covering the entire porous network with evidence of complete pore occlusion in cell culture;* ***b.*** *Cells on chitosan scaffolds characterized by mostly isolated cells a few cell aggregations;* ***c.*** *Cells grown on the surface of 75:25 coralline:chitosan composite scaffolds without sign of pore occlusion.*

4.5. DISCUSSION

Developing scaffolds for bone tissue engineering aim at enhancing mechanical properties while producing highly porous structures to promote cell delivery. This results in conflicting design requirements and goals [46,47]. To conciliate the physical and mechanical requirements, scaffold porosity should be at a maximum as long as the mechanical features are not compromised [48,49]. Although this is a generally accepted concept, the optimal magnitudes of the mechanical requirements of a temporary scaffold are not established [47]. We proposed here, the use of coralline particulate phase to act simultaneously as gas-forming and reinforcing agent to obtain a chitosan-based composite scaffold with improved mechanical and biological properties concomitant with large pores exceeding the upper pore size limit of 250 μm obtained with freeze-dried scaffolds. Choi *et al.* and Chow *et al.* also reported that synthetic $CaCO_3$ as gas-forming agent is highly effective and that the increase of its weight ratio increased the pore diameter and porosity [37,50]. But since a complete reaction occurred and no residual of reinforcing calcium salts was left in the resulting homogeneous material, the largest pore structure was associated with the lowest mechanical strength [37,50].

In the present work, unlike these two reports, the reaction between the coralline $CaCO_3$ and the acidic chitosan solution was rapidly stopped by freezing the samples in the subsequent thermally induced phase separation step (TIPS), leaving coralline particulates to reinforce the polymeric porous structure. As the coralline ratio was raised, the composite suspension became more viscous and consequently the pore wall thickness increased along with a decrease in porosity leading to scaffolds with greater mechanical strength. As a consequence, chitosan-based scaffolds were reinforced proportionally with the addition of coralline particulate phase (Figure 4.2), and the 75:25 coralline:chitosan composite scaffolds provided the largest pore structure with highest compressive properties.

These observations are consistent with those reported earlier with freeze-dried composite polymer scaffolds, where the composites have improved mechanical properties and decreased porosity over those of the pure polymer scaffolds [27,51-53]. However,

contrarily to some other reports, in which the incorporation of particulate weight ratio beyond 50% caused a dramatic decrease of the mechanical strength [10,51], the structural integrity of the composite scaffolds developed in the current study were all reinforced. The homogenous distribution of particulate might contribute to the good integrity of the composite scaffolds in our case, since agglomerations of reinforcing phase have been reported to be potentially detrimental to the compressive properties of composite scaffolds [27].

In addition to above-mentioned structural characteristics, the novelty of this method is that coralline material as gas-forming agent into composite concomitantly created larger pores over both coralline and chitosan scaffolds. Chow *et al.* have reported similar results by obtaining a continuous pore structure from the bulk to the surface of the scaffolds with pore sizes ranging from 100 to 1000 μm depending on the amount of $CaCO_3$ added [37]. Furthermore, as shown by SEM analysis and micro-CT reconstruction imaging (Figures 4.3 and 4.6 respectively), all the coralline:chitosan composites resulted in an open pore macrostructure on the surfaces. Porogen particles, which are the initiators of gas formation, were dispersed into the polymer and caused the evolving gas to permeate from within the bulk to the surface of the material, leaving gas bubbles or pores. However freezing the scaffolds as the CO_2 was released, allowed the matrix porous structures to remain intact after the subsequent lyophilisation process and also contributed to prevent the surface and internal pore to collapse during dehydration process. Our method thus represents an advantage over the gas-foaming technique where the fast diffusion of the gas to the surface collapses the external pores leading to a nonporous surface [30,54].

When the 75:25 coralline:chitosan scaffolds are compared with reference materials, their higher mean porosity (77.85% versus 50%) comparing to coralline might potentially provide higher cell load and attachment enabling presumably faster healing process once implanted into the patient [14]. Conversely, their lower porosity in comparison to chitosan scaffolds provides higher resistance to compression. This might be a suitable compromise since the improvement of scaffold macroporosity, microtopography and

chemistry are parameters known to affect osteogenic cells *in vitro* [36,55] and tissue ingrowth capabilities [27,47].

The increased pore size obtained with the coralline:chitosan composite scaffolds has several advantages over the small pore size of chitosan or coralline scaffolds. We have shown that with an average pore size of 150 μm, complete pore occlusion could occur over the entire porous network of the coralline scaffolds after three weeks of cell culture (Figure 4.8a). Similar results indicated that pore occlusion by cells in biomatrices occurred when the pore size was less than 200 μm [36]. Pore occlusions prevent further cell and tissue ingrowth throughout the scaffold and may affect nutrients circulation and cell metabolites clearance from the scaffolds [36]. Our technique produced composites with an average pore of 400 μm which seems to be in the range of an optimal of pore size. Indeed, previous studies have shown that the nominal pore sizes between 300 to 500 μm [28,36,56] may reduce or obviate the problems of pore occlusion. It could provide curvatures with optimal compression and tension on osteoblast mechanoreceptors, stimulating migration, attachment and proliferation [57], and would allow three-dimensional tissue growth with an optimal bone ingrowth velocity [22,58].

Our SEM observations tend to indicate that the presence of coralline material influence favourably the patterns of cell adhesion/morphology and the biological responses to the different scaffolds. MSCs grown on the pure coralline scaffolds systematically exhibited highly flattened star shape morphology with many projections. As soon as one week of culture, a layer of cell was laid down over the entire porous network. In contrast, on the chitosan scaffolds, only few cells could be observed and the cell morphology remained rounded. The presence of rounded cell on chitosan samples, was in agreement with the previous *in vitro* study reported by Lahiji *et al* who demonstrated that over 90% of the osteoblasts cultured for a period of seven days retained a rounded appearance on a chitosan-coated surface while greater than 90% of these cells grown on uncoated plastic coverslips assumed a spindle shaped, fusiform appearance [59].

These results are differing from several other studies suggesting that chitosan facilitates osteoprogenitor cell adhesion, contributes to their differentiation, and enhances bone formation [23,60.61]. We assume that the low cell density seeding used both by Lahiji *et al.* and in our study [62-65], might explain our results and the delay in the cell spreading on chitosan scaffolds. In fact, cells cultured on both chitosan-coated and uncoated surfaces were randomly distributed at low concentration to avoid cell overlapping and the formation nor formed clusters [59]. Similarly, a low cell-seeding density was performed in our study to allow easier visualization of individual cell interaction with the scaffold material. In both cases, intercellular contacts could not contribute to the cellular regulation.

Nevertheless, the addition of coralline material had a positive impact on the kinetic and degree of adhesiveness as well as on the numbers of pseudopods the cells formed with the substratum. In comparison to the chitosan scaffolds, much more spread cells were observed on the coralline:chitosan scaffolds after one week of culture, most of which were forming pseudopods with coralline particles. Pseudopods developed by cells in response to a stimulus become stabilized and anchored to the substratum through the formation of focal adhesion attachment resulting in structure specifically referred to as focal contacts [66]. Cells forming the highest number of focal contacts have been reported to exhibit a well spread and flattened morphology whereas those with the least number of focal contacts assume a more rounded and less spread shape [67]. Flattened cells by well-defined attachment extensions and several pseudopods, like those observed on the coralline scaffolds, are known to be firmly attached onto surface and have a higher rate of proliferation than cells assuming a rounded morphology as a sign of poor attachment like those observed on the chitosan scaffolds [67-69].

Besides anchoring cells, focal contacts are of enormous importance in the control of cell phenotype, and thus potentially determine the success of the scaffolds [57,67]. By binding to the external environment via integrin receptors, focal contacts play an important role in relaying signals from the material substrates to the cytoskeleton and nucleus, which is affecting the cell shape, gene transcription, and expression of specific phenotypes [69].

The surface texture or microtopography as well as chemistry plays also an important role in the cellular response and adhesion [36,55,58,70,71]. Therefore the rougher topography and different chemistry caused by the presence of coralline in the composite scaffolds have had a positive impact on the formation of pseudopods and by extention potentially on the formation of focal contacts. This represents a relevant feature of our approach since adhesion to substrate is the first step to cell viability, growth, spreading and differentiation [72].

4.6. CONCLUSION

By combining chitosan with different ratios of natural coralline material, which are simultaneously used as particulate reinforcing phase and gas-forming agent, followed by thermally induced phase separation, a family of reinforced macroporous scaffolds with large pore size was developed. Such production of macroporous structures whose integrity is not only maintained but significantly reinforced, suggests that chitosan and coral skeletal material present a great combination to feature an optimized scaffold. With the control over the scaffold parameters such as porosity, pore size, mechanical properties, and cellular affinity, our coralline:chitosan composite scaffolds have demonstrated very interesting structural characteristics justifying our judicious choice of natural coralline biomaterial as a typical particulate reinforcing phase as well as gas-forming agent. Our technique benefits from the chemical reaction between its components that generate CO_2 which contribute to the scaffold's pore morphology. The proposed method is simple, cost-effective, and avoids the use of organic solvents or high temperatures. It requires no additional components or chemicals compared to regular composite scaffolds of polymer and bioceramics that are freeze-dried. The results demonstrate that the kinetic and degree of cellular adhesion were proportional to the coralline content, and may in turn influence favourably the cellular proliferation and differentiation. Our composite scaffolds possess therefore many advantages, such as improved porosity and mechanical properties over coralline and chitosan scaffolds respectively, as well as optimal pore size and biological activities, not only over these two individual homogeneous materials but also comparing to many other scaffolds suggesting

that new coralline:chitosan composites have a great potential as biomatrices for tissue engineering. Extensive *in vitro* experiments are currently ongoing to further investigate the biological response to these novel coralline:chitosan composite scaffolds and to prove the concept.

4.7. ACKNOWLEDGEMENTS

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4.8. REFERENCES

1. Yaszemski MJ, Oldham JB, Lu L, Currier BL. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 541-547.
2. Ohgushi H, Caplan AI. Stem cell technology and bioceramics: from cell to gene engineering. J Biomed Mater Res. 1999;48(6):913-27.
3. Zhao F, Yin Y, Lu WW, Leong JC, Zhang W, Zhang J, Zhang M, Yao K. Preparation and histological evaluation of biomimetic three-dimensional hydroxyapatite/chitosan-gelatin network composite scaffolds. Biomaterials. 2002 Aug;23(15):3227-34.
4. Risbud M. Tissue engineering: implications in the treatment of organ and tissue defects. Biogerontology. 2001;2(2):117-25.
5. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues-state of the art and future perspectives. J Biomater Sci Polym Ed. 2001;12(1):107-24.

6. Laurencin CT, Lu H.H. Polymer-Ceramic Composites for Bone-Tissue Engineering. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 462-468.
7. Gunatillake PA, Adhikari R. Biodegradable synthetic polymers for tissue engineering. Eur Cell Mater. 2003 May 20;5:1-16.
8. Khan SN, Tomin E, Lane JM. Clinical applications of bone graft substitutes. Orthop Clin North Am. 2000 Jul;31(3):389-98.
9. Wang M. Developing bioactive composite materials for tissue replacement. Biomaterials. 2003 Jun;24(13):2133-51.
10. Zhang Y, Zhang M. Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. J Biomed Mater Res. 2001 Jun 5; 55(3): 304-12.
11. Zhang Y, Ni M, Zhang M, Ratner B. Calcium phosphate-chitosan composite scaffolds for bone tissue engineering. Tissue Eng. 2003 Apr;9(2):337-45.
12. Zhang Y, Zhang M. Calcium phosphate/chitosan composite scaffolds for controlled *in vitro* antibiotic drug release. J Biomed Mater Res. 2002 Dec 5;62(3):378-86.
13. Ge Z, Baguenard S, Lim LY, Wee A, Khor E. Hydroxyapatite-chitin materials as potential tissue engineered bone substitutes. Biomaterials. 2004 Mar;25(6):1049-58.
14. Zhang Y, Zhang M. Three-dimensional macroporous calcium phosphate bioceramics with nested chitosan sponges for load-bearing bone implants. J Biomed Mater Res. 2002 Jul; 61(1): 1-8.
15. Linhart W, Peters F, Lehmann W, Schwarz K, Schilling AF, Amling M, Rueger JM, Epple M. Biologically and chemically optimized composites of carbonated apatite and polyglycolide as bone substitution materials. J Biomed Mater Res. 2001 Feb;54(2):162-71.
16. Matthew HW. Polymers for Tissue Engineering Scaffolds. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 170-186.
17. Madihally SV, Matthew HW. Porous chitosan scaffolds for tissue engineering. Biomaterials. 1999 Jun;20(12):1133-42.

18. Suh JK, Matthew HW. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials. 2000 Dec;21(24):2589-98.
19. Domard A, Domard M. Chitosan: Structure-properties relationship and biomedical applications. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 187-212.
20. Dumitriu S. Polysaccharides as biomaterials. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 1-45.
21. Wang JW, Hon MH. Sugar-mediated chitosan/poly(ethylene glycol)-beta-dicalcium pyrophosphate composite: mechanical and microstructural properties. J Biomed Mater Res. 2003 Feb 1;64A(2):262-72.
22. Muzzarelli R, Biagini G, Pugnaloni A, Filippini O, Baldassarre V, Castaldini C, Rizzoli C. Reconstruction of parodontal tissue with chitosan. Biomaterials. 1989 Nov;10(9):598-603.
23. Klokkevold PR, Vandemark L, Kenney EB, Bernard GW. Osteogenesis enhanced by chitosan (poly-N-acetyl glucosaminoglycan) *in vitro*. J Periodontol. 1996 Nov;67(11): 1170-5.
24. Cho BC, Park JW, Baik BS, Kwon IC, Kim IS. The role of hyaluronic acid, chitosan, and calcium sulfate and their combined effect on early bony consolidation in distraction osteogenesis of a canine model. J Craniofac Surg. 2002 Nov;13(6):783-93.
25. Kind GM, Bines SD, Staren ED, Templeton AJ, Economou SG. Chitosan: evaluation of a new hemostatic agent. Curr Surg. 1990 Jan-Feb;47(1):37-9.
26. Khor E, Lim LY. Implantable applications of chitin and chitosan. Biomaterials. 2003 Jun;24(13):2339-49.
27. Yin Y, Ye F, Cui J, Zhang F, Li X, Yao K. Preparation and characterization of macroporous chitosan-gelatin/beta-tricalcium phosphate composite scaffolds for bone tissue engineering. J Biomed Mater Res. 2003 Dec 1;67A(3):844-55.
28. Demers CN, Tabrizian M, Petit A, Hamdy RC, Yahia L. Effect of experimental parameters on the *in vitro* release kinetics of transforming growth factor beta1 from coral particles. J Biomed Mater Res. 2002 Mar 5;59(3):403-10.

29. Patat JL, Guillemin G. Natural coral used as a replacement biomaterial in bone grafts. Ann Chir Plast Esthet. 1989;34(3):221-5.
30. Irigaray JL, Oudadesse H, Blondiaux G. Quantitative study of the coral transformations '*in vivo*' by several physical analytical methods. Biomaterials. 1990 Jul;11:73-4.
31. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. Nat Biotechnol. 2000 Sep;18(9):959-63.
32. Roudier M, Bouchon C, Rouvillain JL, Amedee J, Bareille R, Rouais F, Fricain JC, Dupuy B, Kien P, Jeandot R, Basse-Cathalinat B. The resorption of bone-implanted corals varies with porosity but also with the host reaction. J Biomed Mater Res. 1995 Aug;29(8):909-15.
33. Begley CT, Doherty MJ, Mollan RA, Wilson DJ. Comparative study of the osteoinductive properties of bioceramic, coral and processed bone graft substitutes. Biomaterials. 1995 Oct;16(15):1181-5.
34. Guillemin G, Meunier A, Dallant P, Christel P, Pouliquen JC, Sedel L. Comparison of coral resorption and bone apposition with two natural corals of different porosities. J Biomed Mater Res. 1989 Jul;23(7):765-79.
35. Muzzarelli C, Muzzarelli RA. Natural and artificial chitosan-inorganic composites. J Inorg Biochem. 2002 Nov 11;92(2):89-94.
36. Baksh D. Design strategies for 3-dimensional *in vitro* bone growth in tissue-engineering scaffolds. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 488-495.
37. Chow KS, Khor E. Novel fabrication of open-pore chitin matrixes. Biomacromolecules. 2000 Spring;1(1):61-7.
38. Ravenelle F, Marchessault RH, Légaré A, Buschmann MD. Mechanical properties and structure of swollen crosskinked high amylose starch tablets. Carbohydrate Polymers. 2002 Jan; 47: 259-266.
39. Jurvelin JS, Buschmann MD, Hunziker EB. Optical and mechanical determination of Poisson's ratio of adult bovine humeral articular cartilage. J Biomech. 1997 Mar;30(3):235-41.

40. Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. The use of specific chondrocyte populations to modulate the properties of tissue-engineered cartilage. Orthop Res. 2003 Jan;21(1):132-8.
41. Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. Characterization of cartilagenous tissue formed on calcium polyphosphate substrates *in vitro*. J Biomed Mater Res. 2002 Dec 5;62(3):323-30.
42. Garon M, Legare A, Guardo R, Savard P, Buschmann MD. Streaming potentials maps are spatially resolved indicators of amplitude, frequency and ionic strength dependant responses of articular cartilage to load. J Biomech. 2002 Feb;35(2):207-16.
43. Legare A, Garon M, Guardo R, Savard P, Poole AR, Buschmann MD. Detection and analysis of cartilage degeneration by spatially resolved streaming potentials. J Orthop Res. 2002 Jul;20(4):819-26.
44. Dumont J, Ionescu M, Reiner A, Poole AR, Tran-Khanh N, Hoemann CD, McKee MD, Buschmann MD. Mature full-thickness articular cartilage explants attached to bone are physiologically stable over long-term culture in serum-free media. Connect Tissue Res. 1999;40(4):259-72.
45. http://www.biosensing.com/en/Mach1/A-class.html p.15
46. Claase MB, Grijpma DW, Mendes SC, De Bruijn JD, Feijen J. Porous PEOT/PBT scaffolds for bone tissue engineering: preparation, characterization, and *in vitro* bone marrow cell culturing. J Biomed Mater Res. 2003 Feb 1;64A(2):291-300.
47. Hollister SJ, Maddox RD, Taboas JM. Optimal design and fabrication of scaffolds to mimic tissue properties and satisfy biological constraints. Biomaterials. 2002 Oct;23(20):4095-103.
48. Temenoff, J.S., Lu, L., and Mikos, A.G. Bone-tissue engineering using synthetic biodegradable polymer scaffolds. In: Davies, J.E., ed. Bone Engineering. em sqared incorporated, Toronto, 2000, pp.454-459
49. Caplan, A.I. New logic for tissue engineering: Multifunctional and biosmart delivery vehicules. In: Davies JE, ed. Bone Engineering. em sqared incorporated, Toronto, 2000, pp. 441-445.

50. Choi BY, Park HJ, Hwang SJ, Park JB. Preparation of alginate beads for floating drug delivery system: effects of CO(2) gas-forming agents. Int J Pharm. 2002 Jun 4;239(1-2):81-91.
51. Zhao L, Chang J. Preparation and characterization of macroporous chitosan/wollastonite composite scaffolds for tissue engineering. J Mater Sci Mater Med. 2004 May;15(5):625-9.
52. Ma PX, Zhang R, Xiao G, Franceschi R. Engineering new bone tissue *in vitro* on highly porous poly(alpha-hydroxyl acids)/hydroxyapatite composite scaffolds. J Biomed Mater Res. 2001 Feb;54(2):284-93.
53. Zhang Y, Zhang M. Cell growth and function on calcium phosphate reinforced chitosan scaffolds. J Mater Sci Mater Med. 2004 Mar;15(3):255-60.
54. Harris LD, Kim BS, Mooney DJ. Open pore biodegradable matrices formed with gas foaming. J Biomed Mater Res. 1998 Dec 5;42(3):396-402.
55. Gomi K, Davies JE. Guided bone tissue elaboration by osteogenic cells *in vitro*. J Biomed Mater Res. 1993 Apr;27(4):429-31.
56. Yoshikawa T, Ohgushi H, Tamai S. Immediate bone forming capability of prefabricated osteogenic hydroxyapatite. J Biomed Mater Res. 1996 Nov;32(3):481-92.
57. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials. 1996 Jan;17(2):137-46.
58. Radder AM, Leenders H, van Blitterswijk CA. Application of porous PEO/PBT copolymers for bone replacement. J Biomed Mater Res. 1996 Mar;30(3):341-51.
59. Lahiji A, Sohrabi A, Hungerford DS, Frondoza CG. Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. J Biomed Mater Res. 2000 Sep 15;51(4):586-95.
60. Seol YJ, Lee JY, Park YJ, Lee YM, Young-Ku, Rhyu IC, Lee SJ, Han SB, Chung CP. Chitosan sponges as tissue engineering scaffolds for bone formation. Biotechnol Lett. 2004 Jul;26(13):1037-41.
61. Fakhry A, Schneider GB, Zaharias R, Senel S. Chitosan supports the initial attachment and spreading of osteoblasts preferentially over fibroblasts. Biomaterials. 2004 May;25(11):2075-9.

62. Kii I, Amizuka N, Shimomura J, Saga Y, Kudo A.Cell-cell interaction mediated by cadherin-11 directly regulates the differentiation of mesenchymal cells into the cells of the osteo-lineage and the chondro-lineage. J Bone Miner Res. 2004 Nov;19(11):1840-9. Epub 2004 Aug 23.
63. Ferrari SL, Traianedes K, Thorne M, Lafage-Proust MH, Genever P, Cecchini MG, Behar V, Bisello A, Chorev M, Rosenblatt M, Suva LJ. A role for N-cadherin in the development of the differentiated osteoblastic phenotype. J Bone Miner Res. 2000 Feb;15(2):198-208.
64. Schiller PC, D'Ippolito G, Balkan W, Roos BA, Howard GA. Gap-junctional communication is required for the maturation process of osteoblastic cells in culture. Bone. 2001 Apr;28(4):362-9.
65. Cheng SL, Lecanda F, Davidson MK, Warlow PM, Zhang SF, Zhang L, Suzuki S, St John T, Civitelli R. Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation. J Bone Miner Res. 1998 Apr;13(4):633-44.
66. Frame MC, Fincham VJ, Carragher NO, Wyke JA. v-Src's hold over actin and cell adhesions. Nat Rev Mol Cell Biol. 2002 Apr;3(4):233-45.
67. Hunter A, Archer CW, Walker PS, Blunn GW. Attachment and proliferation of osteoblasts and fibroblasts on biomaterials for orthopaedic use. Biomaterials. 1995 Mar;16(4):287-95.
68. Rothamel D, Schwarz F, Sculean A, Herten M, Scherbaum W, Becker J. Biocompatibility of various collagen membranes in cultures of human PDL fibroblasts and human osteoblast-like cells. Clin Oral Implants Res. 2004 Aug;15(4):443-9.
69. Trylovich DJ, Cobb CM, Pippin DJ, Spencer P, Killoy WJ. The effects of the Nd:YAG laser on *in vitro* fibroblast attachment to endotoxin-treated root surfaces. J Periodontol. 1992 Jul;63(7):626-32.
70. Baxter LC, Frauchiger V, Textor M, ap Gwynn I, Richards RG. Fibroblast and osteoblast adhesion and morphology on calcium phosphate surfaces. Eur Cell Mater. 2002 Sep 30;4:1-17. Print 2002 Sep 30.

71. Bowers KT, Keller JC, Randolph BA, Wick DG, Michaels CM. Optimization of surface micromorphology for enhanced osteoblast responses *in vitro*. Int J Oral Maxillofac Implants. 1992 Fall;7(3):302-10.
72. Lee JW, Kim YH, Park KD, Jee KS, Shin JW, Hahn SB. Importance of integrin beta1-mediated cell adhesion on biodegradable polymers under serum depletion in mesenchymal stem cells and chondrocytes. Biomaterials. 2004 May;25(10):1901-9.

CHAPTER 5: Responses of Mesenchymal Stem Cell to Novel Chitosan-Coralline Composites Microstructured Using Coralline as Gas Forming Agent

The purpose of this study was to examine novel coralline:chitosan macroporous composite scaffolds for their supportive activity of cellular attachment, proliferation and differentiation. Five different experimental groups of scaffolds were studied consisting of three groups of coralline:chitosan composites containing different ratios of coralline material (25, 50, and 75 wt%) that were developed and compared against pure chitosan and pure coral scaffolds as reference materials.

The results obtained in objective 2 of this thesis, presented in the chapter 4, have demonstrated a clear evidence of the effect of coral content on the physical properties of the scaffolds. It was observed that higher coral proportions increased pore sizes and pore wall thickness, decreased porosity, and improved the mechanical strength of the scaffold. Moreover, the increasing density of coral particles on the surface with higher coral ratios changed considerably the smooth surface morphology of pure chitosan.

Therefore, since, *in vitro*, several factors influence the osteogenic cells responses to scaffolds including the macroporosity, surface microtopography, and chemistry [1-3], the biological response to the different group of scaffolds are expected to differ.

Moreover, the preliminary results of cell culture previously examined by SEM in the objective 3a presented also in the chapter 4 of this thesis, seemed to be consistent with this hypothesis, and showed changes in cell adhesion and morphology in response to the different substrates tested. The results were promising and tended to indicate that the scaffolds containing coral offered surface conditions allowing faster cell attachment and spreading compared to pure chitosan scaffolds. It is thus reasonable to expect that these substrates would also affect proliferation and differentiation of cells.

Therefore, in the present study, in order to pursue the optimisation process and ascertain the findings of the preliminary *in vitro* study, the scaffolds were cultured with MSCs, examined for cell adhesion, morphology and proliferation as well as for the development of the osteoblast phenotype markers: ALP enzyme activity, and expression of the bone matrix protein osteocalcin. The effect of cell culture medium supplementation with β-glycerophosphate and dexamethasone on the osteogenic phenotype of these cells was studied in relation with the type of scaffolds on which the cells were growing.

The results of the objectives 3 and 4 mentioned above have been reported in a manuscript that has been accepted for publication in the journal of "Biomaterials".

This manuscript is entitled: "Responses of Mesenchymal Stem Cell to Chitosan-Coralline Composites Microstructured Using Coralline as Gas Forming Agent", by Mylène Gravel, Talia Gross, Razi Vago, Maryam Tabrizian.

REFERENCES

1. Gomi K, Davies JE. Guided bone tissue elaboration by osteogenic cells *in vitro*. J Biomed Mater Res. 1993 Apr;27(4):429-31.
2. Baksh D. Design strategies for 3-dimensional *in vitro* bone growth in tissue-engineering scaffolds. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 488-495.
3. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials. 1996 Jan;17(2):137-46.

5. PAPER 2

Responses of Mesenchymal Stem Cell to Chitosan-Coralline Composites Microstructured Using Coralline as Gas Forming Agent

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5.1. ABSTRACT

Macroporous composites made of coralline:chitosan with new microstructural features were studied for their scaffolding potential in *in vitro* bone regeneration. By using different ratios of natural coralline powder, as *in situ* gas forming agent and reinforcing phase, followed by freeze-drying, scaffolds with controlled porosity and pore structure were prepared and cultured with mesenchymal stem cells (MSCs). Their supportive activity of cellular attachment, proliferation and differentiation were assessed through cell morphology studies, DNA content, alkaline phosphatase (ALP) activity and osteocalcin release. The coralline scaffolds showed by far the highest evaluation of cell number and ALP activity over all the other chitosan-based scaffolds. They were the only material on which the osteocalcin protein was released throughout the study. When used as a component of the chitosan composite scaffolds, these coralline's favourable properties seemed to improve the overall performance of the chitosan. Distinct cell morphology and osteoblastic phenotype expression were observed depending on the coralline to chitosan ratios composing the scaffolds. The coralline-chitosan composite scaffolds containing high coralline ratios generally showed higher total cell number, ALP activity and

osteocalcin protein expression comparing to chitosan scaffolds. The results of this study strongly suggest that coralline:chitosan composite, especially those having a high coralline content, may enhance adhesion, proliferation and osteogenic differentiation of MSCs in comparison with pure chitosan. Coralline:chitosan composites could therefore be used as attractive scaffolds for developing new strategies for *in vitro* tissue engineering.

Keywords: Composite scaffolds, chitosan, coralline, *in situ* gas forming agent, freeze-drying, controlled microstructural features, mesenchymal stem cells, cell-material interaction.

5.2. INTRODUCTION

The design and selection of scaffolding biomaterials can significantly affect the development of engineered tissues [1]. An ideal scaffold must be biocompatible, possess sufficient transient mechanical properties, highly porous having an interconnected pore network, with surface chemistry that promotes an appropriate regulation of cell behaviour such as cell adhesion, proliferation, migration and differentiation. It is favourable that degradation and resorption kinetics match tissue formation *in vitro* / *vivo* to maintain its structural integrity [2-4].

Finding such material still remains a major challenge in the field of tissue engineering, since no homogeneous material currently provides these essential features [5-7]. The combination of appropriate organic and inorganic materials provides an alternative choice to combine the best properties of each phase while overcoming many of their shortcomings when used as homogenous materials [5,8-10].

Among homogeneous materials, chitosan [8,11] and coralline derivative materials [12,13] have been reported as promising candidates for bone repair and regeneration. Due to its structure analogous to glycosaminoglycans and cationic nature in physiological pH, chitosan exhibits multiple biological activities [11,14,15]. It is biocompatible,

biodegradable, bioresorbable and its hydrophilic nature facilitates cell adhesion, proliferation, and differentiation [14,16-18]. Chitosan showed promising results for engineering numerous tissue systems [8,11]. Moreover, chitosan has excellent ability to be processed into porous structures [11]. However, although chitosan has very attractive properties, it lacks bioactivity and it is mechanically weak [3,19].

Conversely, the exoskeletons edified by some coralline species possess very important qualities for bone substitution: they have inherent resorbability, osteoconductivity and adequate initial mechanical properties [20-23]. With their chemical composition similar to those of human bones [20,24-28], they are considered as biocompatible materials [29-32], and also have been proven to be bioactive [20,33]. Since 1980, natural coralline, made of calcium carbonate ($CaCO_3$), and its converted hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, had been widely used as a replacement biomaterial for bone grafts in different surgical specialties [20,34-37].

We have recently demonstrated that coralline could simultaneously be used as particulate reinforcing phase and gas-forming agent by being incorporated into the chitosan polymer [38]. A reaction between the calcium carbonate and acetic acid, which is the chitosan polymer solvent, generates carbon dioxide leading to growth of large gas pores. Consequently, a reinforced porous structure of large pore size (80 μm versus 400 μm) is achieved by increasing the coralline weight ratio from 0 to 75 wt%.

The introduction of the coralline particulate phase into the chitosan polymer has also modified the surface microtopography and the scaffold macroporosity. These parameters are known to influence the behaviour of osteogenic cells *in vitro* [39-41]. We hypothesized that the biological response to the composite scaffolds may be modified due to changes of those parameters as well as due to the strong biological activity of chitosan [26] along with that of aragonite crystals, offering potentially an improved cells delivery and control over their differentiation [42,43].

In order to confirm this hypothesis, we investigated responses of MSCs to these novel three-dimensional coralline-chitosan macroporous composite scaffolds. Chitosan-based hydrogel scaffolds varying in coralline material contents (0, 25, 50, 75, and 100 wt%) were fabricated and cultured with MSCs. Cell morphology, proliferation, ALP activity and osteocalcin production were monitored. The effect of cell culture medium supplementation with β-glycerophosphate and dexamethasone on the osteogenic phenotype of these cells was studied in relation with the type of scaffolds on which the cells were grown.

5.3. MATERIALS AND METHODS

5.3.1. Scaffold production and preparation

The preparation and characterization of scaffolds were previously reported [38]. Briefly, chitosan solution with a concentration of 2 wt% was prepared by dissolving high viscosity chitosan (1186 cPs, MW range from ~300kDa - 800kDa with a deacetylation degree of 84.9, Vanson HaloSource, WA) in a 0.2 M acetic acid.

Coral skeletal material was obtained using small coral cores, from the species *Porites lutea,* harvested at the Seychelles archipelago. Exoskeleton blocs had an average pore size of 150 μm and an open porosity between 47-51%. They were soaked in NaOH 2N for 5 min, then treated with H_2O_2 30% for 10 min to remove trapped particles, debris and organic remnants. They were then broken in small pieces and shaped with sand paper to obtain flat squared coralline scaffolds. The prepared coralline scaffolds were rinsed with distilled water and sterilized with an autoclave at 121°C for 40 min and oven-dried overnight at 80°C.

For composite scaffold preparation, the coralline powder was obtained by milling solid coralline material for 8 h in a rotating micromill. The powder was then sieved through 20 um U.S. standard sieve. Different proportions of coralline powder (25, 50, and 75 wt%) were added into the prepared chitosan solution. The mixture was then continuously stirred until a homogeneous slurry was obtained. Pure chitosan and the mixture were poured into

96 multi-well polystyrene culture dishes and rapidly transferred into a freezer at –80°C overnight to induce a solid-liquid phase separation. They were then freeze-dried at a temperature of –52°C for 4 days.

After lyophilisation, samples were neutralized by immersing them in absolute ethanol for 1h, followed by ethanol 70%/water (v/v) and 50% for 30 min each. The pH was adjusted with phosphate buffered saline (PBS) to 7.4 and scaffolds were rinsed with double-distilled water. The hydrated samples were frozen at –80°C, and cut with a razor blade into disks. The scaffolds were freeze-dried and kept in a closed environment until use. Prior to cell seeding, the scaffolds were sterilized with STERRAD ® 200 System (Jonhson & Jonhson).

5.3.2. Cell culture and proliferation

MSC's (mouse cell line CRL-12424, ATCC, Manassa, VA, USA), were cultured for 5 passages in Dulbecco's modified Eagle medium (DMEM, Gibco, Burlington, ON, Canada), supplemented with 4 mM-glutamine, 4.5 g/L glucose, and 25 mM HEPES buffer, 10% FBS (Gibco), 10 U/ml penicillin G sodium, 10 μg/ml streptomycin, and 25 μg/ml amphotericin B as Fungizone (Gibco), and 100 μg/ml L-ascorbic acid (Sigma-Aldrich, Oakville, ON, Canada).

Prior to cell seeding, the scaffolds were sterilized with STERRAD ® 200 System (Jonhson & Jonhson). From the seeding time, half of the cell-loaded scaffolds were cultured in the presence of 100 nM dexamethasone (Sigma) and 10 mM β-glycerophosphate (Sigma) in the medium to study the effect of these osteogenic supplements on cell proliferation and differentiation. Aliquots of 40 μl cell suspension were seeded onto the top of each dry scaffolds placed in 48 multi-well plates resulting in a seeding density of $5x10^5$ cells/cm^2. 150 μl of medium were added into each well. The scaffolds were incubated for 12 h to allow cells to attach to surface of scaffold. They were then transferred into 35 mm tissue culture polystyrene dishes and cultured for 3, 7, 14, 21 and 28 days. Media were changed every 2-3 days.

5.3.3. Cell morphology

The morphology of cells was examined with SEM (Hitachi field emission s-4700). The cell-seeded scaffolds were rinsed twice with PBS, and fixed with 2.5% glutaraldehyde in 0.14M sodium cacodylate (pH 7.4) for 24h at 5°C and dehydrated consecutively in 30, 50, 70, 80, 90, and 100% ethanol for 10 min. Further, substitution to amyl acetate was done through 4 graded bath of amyl acetate:ethanol (25:75), followed by (50:50), (75:25), and (100%). Samples were then critically point dried and covered with a thin layer of gold-palladium through sputtering under an argon atmosphere prior to SEM observation.

5.3.4. Cell counting assay

The cell quantification was performed on days 3, 7, 14, and 35 with fluorometric quantification of DNA using CyQuant® Cell Proliferation Assay Kit (Molecular Probes, Leiden, NL) for DNA content. In order to assess the cell counting in the same conditions, scaffolds were washed with PBS at each time point and frozen at −80°C until the analysis.

The cyquant GR dye was diluted 1:80 for 5x final concentration into lysis buffer. When the samples were thawed, 200 µl of the Cyquant® lysis buffer was added to each scaffold and the lysate was resuspended by brief vortexing once the scaffolds were cut in at least four pieces. For each sample, aliquots of 200 µl were transferred to a solid black 96-faltted-bottom-well plate (Costar). After a short incubation in the dark, the fluorescence was measured with a µQuant spectrophotometer FLX800 microplate fluorescence reader (Bio-Tek Instruments, Inc. Vermont USA). Cell numbers in the scaffolds were determined by comparing the sample fluorescent results to the cell standard curves.

For preparation of cell standards, aliquots containing $1.25x10^6$ cultured cells were assessed from cell suspensions with a hemocytometer. After centrifugation at 125 000 g for 10 minutes, the supernatant was removed and the resulting cell pellets were stored in a freezer at -80°C. When the standards were thawed at room temperature, 1000 µl of the cyquant GR dye 5x in cell lysis buffer was added to each pellet and the lysate was resuspended by brief vortexing. A cell dilution series ranging from 50 to 250 000 cells

was prepared in triplicate from the standard pellet with Cyquant® lysis buffer. For each cell standard dilution, 3 aliquots of 200 µl each were transferred to a well. A standard calibration curve was generated by plotting measured fluorescence values versus cell number.

5.3.5. ALP activity

ALP activity was determined on days 3, 7, 14, 21, and 28, by using a phosphatase substrate kit (Pierce Chemicals, Co., Rockford, IL, USA) by adapting a similar protocol to Zhang's *et al.* [3]. The seeded scaffolds were rinsed twice with cold PBS and transferred individually into test tubes. 1 ml of cold Triton X-100 (0.2% in PBS) was added before being vortexed for 30 sec. After being left undisturbed for 10 min at 4°C, the foams were vortexed for 30 sec. Samples were sonicated for 10 min in ice-cold water and vortexed again for 30 sec. Three different dilutions 1:1, 1:3, and 1:6 (v/v) of Triton lysate containing the enzyme were prepared with Triton X-100 (0.2%). p-nitrophenylphosphate (p-NPP) was added into the each dilution in at ratio of 1:2 (v/v). The blank sample was composed with 400 µl of Triton X-100 (0.2% in PBS) and 200 µl of P-NPP). The samples were then incubated at 37°C for 45 min. After incubation, 50 µl of 2N sodium hydroxide was added to stop the reaction. The release of the coloured product p-nitrophenol (p-NP) is measured spectroscopically from absorbance at 405 nm using previously mentioned microplate reader.

5.3.6. Osteocalcin

Osteocalcin levels were determined on days 7, 14, and 35, by using an enzyme-linked immunosorbent assay kit (ELISA, Biomedical Technologies, Stoughton, MA). After each period of time in culture, the seeded scaffolds were transferred into a 96 multi-well plate with 200 µl of complete cell culture medium containing no FBS. After incubation at 37°C for 24 hours, 25 µl of cell culture supernates were tested. The osteocalcin level was expressed as nanograms per ml.

5.3.7. Statistical analysis

Various numbers of samples were collected for DNA, ALP, and osteocalcin measurements. Data were reported as means ± standard deviations. One-way analysis of

variance (ANOVA) with Tukey's multiple comparison tests was used to assess the statistical significance among the data obtained for different groups of samples.

5.4. RESULTS

5.4.1. Cell proliferation

Whether the cells were cultured in absence or in presence of the osteogenic supplemented medium, similar trend in cell number were obtained (Figure 5.1). Cell proliferation process on the coralline material scaffolds was high and fast at the beginning, bringing the cell number to a level significantly higher ($p<0.001$) than all the chitosan-based scaffolds.

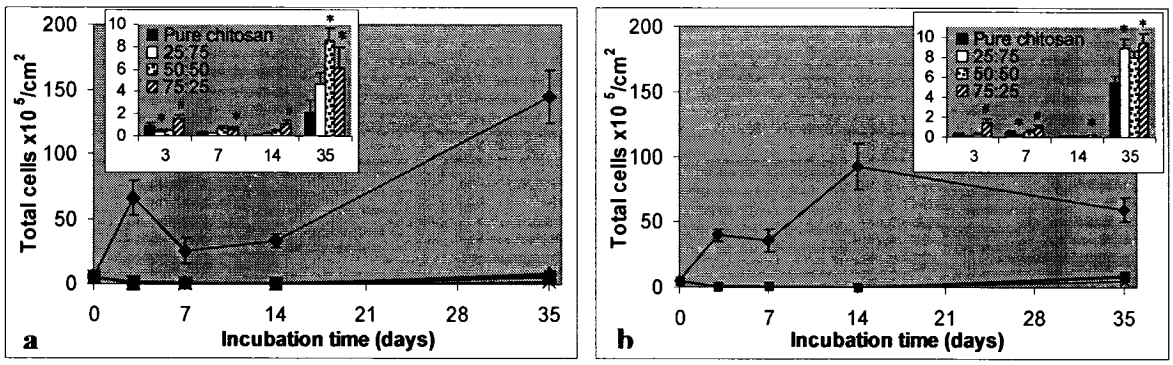

Figure 5.1. Total number of MSCs per cm2 cultured up to 35 days on porous scaffolds containing varying amount of chitosan and coralline material. *(a) Cell culture in absence of osteogenic supplements dexamethasone and β-glycerophosphate following seeding. (b) Cell culture supplemented with osteogenic supplements. Pure coralline (♦), pure chitosan (×), 25:75 (●), 50:50 (▲), and 75:25 (■) coralline:chitosan composites. Insets show a histogram representing the cell number of the chitosan-based scaffolds only. Values represent the mean± SD of four samples. *p<0.05, any chitosan-based composite scaffolds versus pure chitosan scaffolds; #significantly different (p<0.05) than all the other chitosan-based scaffolds.*

As opposed to the fast increase in cell number observed with the coralline scaffolds, the number of cells cultured on the chitosan-based scaffolds significantly decreased in the first three days of the culture. For the latter, the resulting number remained at a low level during fourteen days before increasing significantly at thirty five days of culture. The chitosan scaffolds were generally containing the lowest cell numbers comparing to other composite scaffolds, especially comparing to the 75:25 coralline:chitosan composites that were possessing significantly higher cell number.

5.4.2. ALP activity

ALP activity of the cells was also similar in absence or in presence of the osteogenic supplemented medium (Figure 5.2). The ALP activity of the cells grown on the coralline scaffolds was exponentially increasing and was significantly higher (generally $p<0.001$) than all the chitosan-based scaffolds.

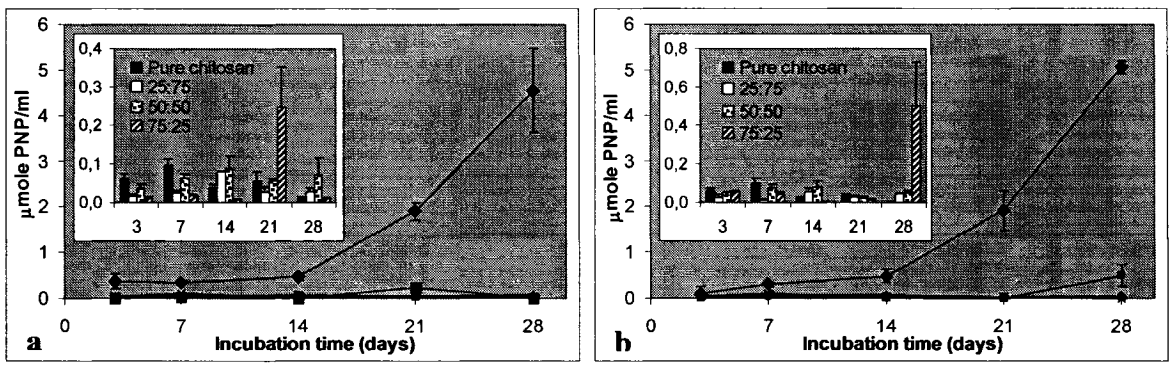

Figure 5.2. ALP activity of MSCs cultured for 28 days on porous scaffolds containing varying amount of chitosan and coralline material. *(a) Cell culture in absence of osteogenic supplements dexamethasone and β-glycerophosphate following seeding. (b) Cell culture supplemented with osteogenic supplements. The ALP activity of the cells grown on the pure coralline scaffolds was significantly higher ($p<0.001$). Pure coralline (♦), pure chitosan (×), 25:75 (●), 50:50 (▲), and 75:25 (■) coralline:chitosan composites. Inset shows a histogram representing the ALP activity of the chitosan-based scaffolds in comparison to chitosan alone. Error bars represent means standard deviations for n = 5.*

The ALP activities for the four families of chitosan-based scaffolds were similar up to two weeks. A significant increase ($p<0.001$) of ALP activity could be noticed for 75:25 coralline:chitosan at twenty one days in the absence of osteogenic supplemented medium and after twenty eight days in the presence of osteogenic supplemented medium.

5.4.3. Osteocalcin expression

The coralline scaffolds were the only materials on which the osteocalcin protein was released throughout the study and generally at a high level. In fact, on the chitosan-based scaffolds, the osteocalcin expression only significantly increased after more than fourteen days of cell culture. Thirty five days after the seeding, the osteocalcin protein was expressed by all the five cultured groups of scaffolds and reached its highest level. Interestingly, cell seeded on the 75:25 coralline-chitosan scaffolds would be able to release osteocalcin at a level comparable to the coralline samples in presence or absence of osteogenic supplements.

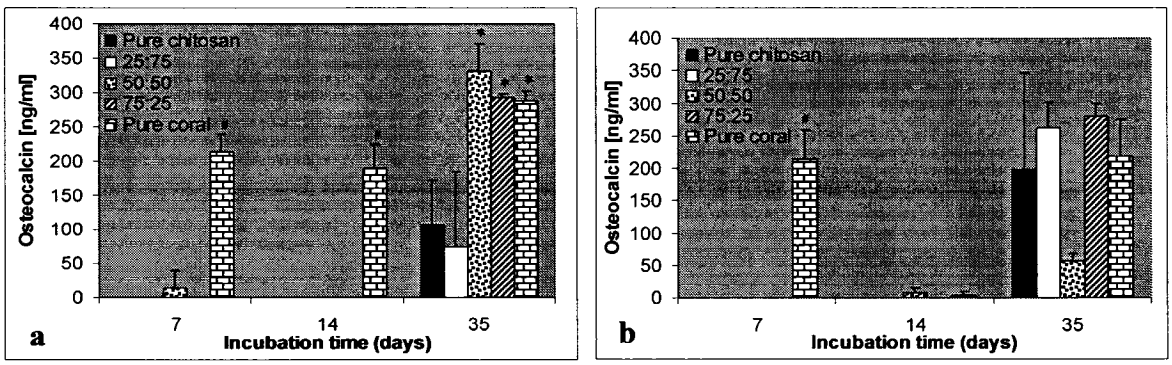

Figure 5.3. Osteocalcin production by MSCs seeded on pure chitosan, 25:75, 50:50, 75:25 coral:chitosan composites, and coralline scaffolds. *Cells were cultured for 35 days: (a) in absence or (b) in presence of osteogenic supplements dexamethasone and β-glycerophosphate following seeding. Values represent the mean± SD of three samples.* $*p<0.05$, *any of the 4 group of scaffolds containing coralline versus pure chitosan scaffolds;* $^{\#}$*significantly different* ($p<0.05$) *than all the other group of scaffolds.*

In the presence of the osteogenic supplemented medium, no specific trend in osteocalcin production was observed depending on the type of chitosan-based scaffolds on which the cells were grown (Figure 5.3b). However, in absence of the osteogenic supplemented medium (Figure 5.3a), the osteocalcin protein expression by coralline and by the scaffolds having high coralline content, namely, 50:50 and 75:25 coralline:chitosan scaffolds, thirty five days after the cell seeding, were all significantly higher ($p<0.05$) than the level simultaneously observed on the chitosan and the 25:75 coralline:chitosan scaffolds.

5.4.4. SEM analysis

After three days of culture, while the cells on pure coralline scaffold surfaces exhibited a highly flattened shape with an elongated cell body, intercellular communication, numerous microspikes and filopodia (Figure 5.4a), the cells generally remained rounded (Figure 5.4b), and numerous dead cells were found on the chitosan-based scaffolds.

After one week of culture, a large number of cells were found on the pure coralline scaffolds, sometimes forming a uniform layer and beginning to bridge over pores. On the coralline-chitosan composite scaffolds, more cell-spreading was observed in comparison with the third day of culture. The cells generally assumed a larger cell body with signs of dorsal activity with an increased number of pseudopods. However, semi-spread cells and rounded cells were still present, particularly on the chitosan scaffolds, on which the majority of cells were exhibiting a rounded shape. Figure 5.5 depicts such typical cell-material interactions on both chitosan and coralline-chitosan samples.

After two weeks of culture, the cell density was quite different among the three categories of scaffolds. As a sign of good biocompatibility, a thick layer of cells composed of a dense extracellular matrix was observed on the coralline scaffolds which caused the occlusion of the majority of pores. Contrarily, on the chitosan scaffolds, the progression of cellular activities as they could be revealed by SEM analysis, were very slow. Some cell spreading and evolvement in cell morphology could be observed with sign of activity such as microvilli and fibre-like formations, but even after 4 weeks of culture, only cell aggregation were found on the surface of chitosan scaffolds (Figure 5.6a).

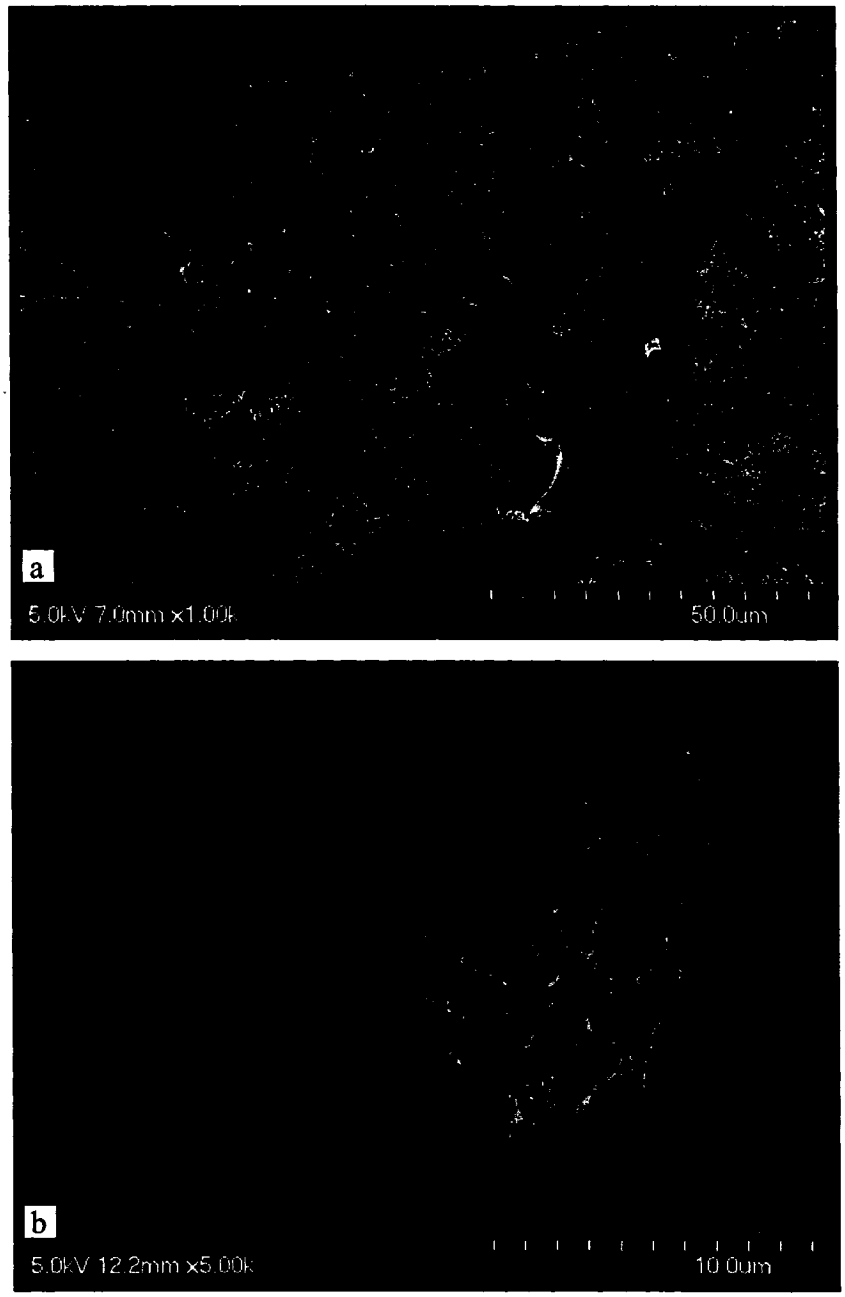

Figure 5.4. SEM micrographs showing distinct differences in morphology between cells on coralline and chitosan-based scaffolds after three days of culture. *(a) Cells on the rough surface of coralline exhibited a flattened morphology with an elongated cell body, high dorsal cell surface activity, numerous pseudopodial and filopodial extensions, and formed intercellular communications. Original magnification x1k. (b) General rounded cell morphology observed for more than two weeks on smooth chitosan surfaces. Original magnification x5k.*

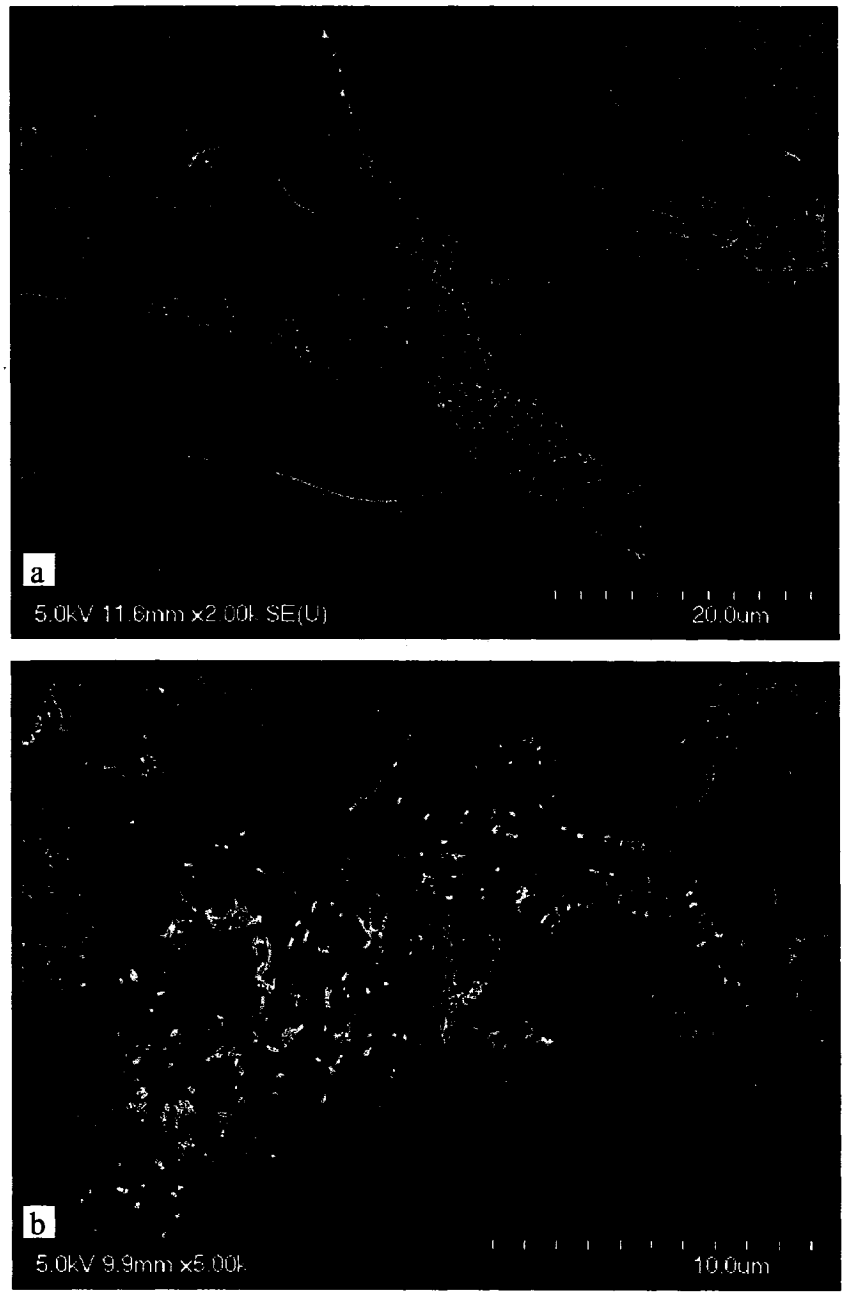

Figure 5.5. SEM micrographs of spread cells with different degrees of cell-substrate adhesion. *(a) On pure chitosan scaffolds. Original magnification 2k. The upper inset shows a 10k high-magnification of filopodia. (b) The formation of numerous pseudopodial and filopodial extensions seemed to be stimulated on the 75:25 coralline:chitosan composite scaffolds. Original magnification 5k.*

The cell spreading and morphology on the other three composite scaffolds become similar to those of coralline samples for a longer period of cell culturing as the ratio of the coralline material increased in the scaffold composition. After four weeks, the cell on the 75:25 coralline:chitosan composite scaffolds were at the similar stage as observed for coralline materials. A dense cell layer was covering the surfaces but interestingly it was not causing pore occlusion (Figure 5.6b). After this period, the surface of coralline scaffolds was however characterized by multiple dense layers of active cells over the entire porous network causing complete pore occlusions (Figure 5.6c).

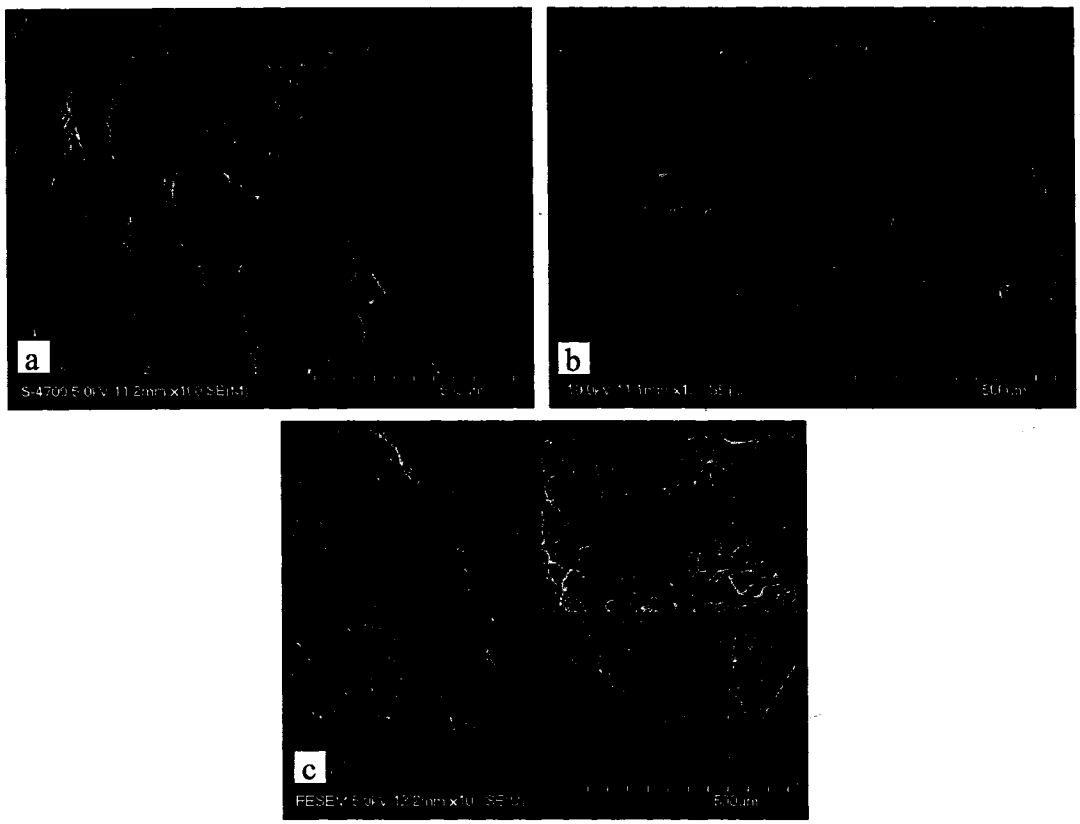

Figure 5.6. SEM micrographs of samples cultured for 4 weeks with mice MSCs. *Original magnifications x100. Figure (a) shows the few cells growing on the edge of pure chitosan scaffolds. (Inset x1k). Figure (b) shows a dense cell layer covering the surface of 75:25 coralline:chitosan composite scaffolds without signs of pore occlusion or cell bridging. Cells were growing inside the pores (Inset x1k). Figure (c) shows multiple dense layers of cells which have been laid down over the entire porous network and caused complete pore occlusions. (Inset x5k).*

5.5. DISCUSSION

Consistent with our initial hypothesis, distinct cell density, ALP activity, and osteocalcin release were observed depending on the type of material on which the cells were grown. Striking results were obtained for coralline scaffolds, on which the cell number and ALP activity were remarkably higher than those obtained for chitosan-based scaffolds. Moreover, the coralline scaffolds were the only material on which the cells produced a high level of osteocalcin protein throughout the study. Also, SEM observation tended to indicate that coralline scaffolds offered surface conditions allowing the fastest cell attachment and the highest degree of cell spreading among the biomaterials used in the present study. All these observations are in agreement with a recent work demonstrating the osteogenic capacity of two kinds of aragonite crystalline derivatives of marine origin [44]. They also concord with previously reported results, suggesting that coralline materials facilitate cell proliferation, induce a rapid cell differentiation [43,45], and maintain differentiated phenotype including the expression of high ALP activity and osteocalcin even after five weeks of culture [29].

Although such performance of natural coral exoskeleton comforts our selection of this biomaterial, the originality of our approach relies on using this material as reinforcing and gas forming agent in the preparation of chitosan-based scaffolds to take advantages of the coralline biomaterial bioactivity while avoiding its limitations. The advantage of using this strategy was fourfold: control of scaffold microarchitecture which is not possible with the coralline materials; avoiding the complete pore occlusion which prevent cell and tissue ingrowth as could occur with coralline materials; improvement of chitosan mechanical properties and promoting the osteoblastic cell activities while taking advantage of chitosan biological activities and finally, timely monitoring of the cell ingrowth by varying the coralline ratio in our scaffolds' composition.

Indeed our results showed that the addition of coralline to chitosan improved the overall properties of the composites and greatly enhanced the osteoblasts turnover. Composites containing high coralline ratios significantly increased the cellular response in terms of

morphology, proliferation, ALP activity and osteocalcin protein expression in comparison with the chitosan scaffolds.

Conversely, as shown in Figure 5.6, the addition of 75% by weight of coralline powder to the chitosan biomatrices led to scaffolds having an average pore size of 400 μm. This could overcome the pore occlusion that occurred with coralline scaffolds possessing an average pore size of 150 μm. In addition, using lower ratios of coralline showed to delay the cellular activities. This would allow monitoring the cellular activities which might be beneficial for developing programmable tissue engineering strategies.

The difference in cell proliferation and differentiation reported in this work could be related to the distinct degree of cell adhesion observed on the scaffolds. According to the literature, the degree of cell adhesion to a substrate can be reflected through the cell morphology and ultimately through the phenotypic expression of cells [40,46]. Our microscopic analyses suggested that the introduction of the coralline particulate phase into the chitosan scaffolds have influenced positively the patterns of cellular adhesion and morphology. The acceleration in the formation of numerous pseudopodial and filopodial extensions and faster cell spreading after one week on the coralline-chitosan composites scaffolds could be correlated to a better cell affinity and behaviour in comparison to chitosan matrices on which this spreading was only initiated after two weeks of cell seeding.

This better performance obtained by the scaffolds containing coralline material, may be attributed in part to the cell-substrate interactions mediated by surface chemistry and topography [39,41,47]. Since coralline material exhibits a crystalline configuration, having an aragonite needle shape elongated crystal [48], one could conclude that these particles provide better adhesion sites for the cells than the chitosan alone. Furthermore, some recent findings suggest that cells grown onto the coralline aragonite crystals may directly exploit calcium ions of the biomaterial which in return affect their adhesion and differentiation [49]. The rougher topography generated by the presence of coralline particulates of varying sizes (smaller than 20 microns) on the composites scaffolds, have

also presumably played a role in improving the osteoblastic cell attachment, proliferation and differentiation of our composite scaffolds [50]. According to the literature, rough surfaces appear to stimulate the formation of focal adhesions and focal contacts that allow the cells to span across the space between surface peaks [40,51,52].

Focal contacts occur through discrete regions of a cell membrane binding to the external environment via integrin receptors [40,45,53]. Variation in focal contacts have been shown to result in new gene transcription, and new protein synthesis, ultimately affecting phenotypic expression [40]. Consequently, besides anchoring cells, focal contacts play an important role in relaying signals from the material substrates to the actin cytoskeleton, which is affecting the cell shape [40,45]. The signal is also further transduced via the cytoskeleton to the nucleus, resulting in new gene transcription and expression of specific phenotypes [40].

Our findings for the cell quantification assays are in accordance with the above mentioned hypothesis which relates that flattened cells, as the one observed on coralline scaffolds, are firmly attached onto surface and have a higher rate of proliferation. Contrarily, a rounded cell morphology, as the one observed on the chitosan scaffolds, is considered as a sign of poor attachment to the substrate, forming less focal adhesions and consequently would correspond to a lower degree of cell proliferation and differentiation [54-56].

In addition to the cell-substrate interactions, other extracellular stimuli including the presence of soluble inducers have also been proposed to regulate proliferation, differentiation, and metabolic activity of osteoblastic lineage cells *in vitro* [40,45,57,58]. Exposing the undifferentiated cell in culture to osteogenic supplements such as ascorbic acid, β-glycerophosphate, and dexamethasone has been widely reported to direct the stem cell's differentiation towards the osteoblast lineage [20,59-61]. Interestingly, in the present study, no general tendency seemed to be followed by cells in response to osteogenic supplements. These results tend to suggest that the osteogenic supplements

may not be an absolute requirement for the expression of osteogenic markers of the cell line, at least for the five groups of scaffolds used in this study.

5.6. ACKNOWLEDGEMENT

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5.7. REFERENCES

1. Godbey WT, Atala A. *In vitro* systems for tissue engineering. Ann N Y Acad Sci. 2002 Jun; 961: 10-26.
2. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues-state of the art and future perspectives. J Biomater Sci Polym Ed. 2001;12(1):107-24.
3. Zhang Y, Zhang M. Three-dimensional macroporous calcium phosphate bioceramics with nested chitosan sponges for load-bearing bone implants. J Biomed Mater Res. 2002 Jul; 61(1): 1-8.
4. Temenoff JS, Lu L, Mikos AG. Bone-tissue engineering using synthetic biodegradable polymer scaffolds. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp.454-459.
5. Laurencin CT, Lu H.H. Polymer-Ceramic Composites for Bone-Tissue Engineering. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 462-468.
6. Gunatillake PA, Adhikari R. Biodegradable synthetic polymers for tissue engineering. Eur Cell Mater. 2003 May 20;5:1-16.
7. Khan SN, Tomin E, Lane JM. Clinical applications of bone graft substitutes. Orthop Clin North Am. 2000 Jul;31(3):389-98.
8. Madihally SV, Matthew HW. Porous chitosan scaffolds for tissue engineering. Biomaterials. 1999 Jun;20(12):1133-42.

9. Ramakrishna S, Mayer J, Wintermantel E, Leong KW. Biomedical application of polymer-composite materials: A review. Composites Science and Technology. 2001 Jul;61(9):1189-1224.
10. Wang M. Developing bioactive composite materials for tissue replacement. Biomaterials. 2003 Jun;24(13):2133-51.
11. Matthew HW. Polymers for Tissue Engineering Scaffolds. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 170-186.
12. Gao TJ, Lindholm TS, Kommonen B, Ragni P, Paronzini A, Lindholm TC, Jalovaara P, Urist MR. The use of a coral composite implant containing bone morphogenetic protein to repair a segmental tibial defect in sheep. Int Orthop. 1997;21(3):194-200.
13. Soost F. Biocoral an alternative bone substitute. Chirurg. 1996 Nov;67(11):1193-6.
14. Domard A, Domard M. Chitosan: Structure-properties relationship and biomedical applications. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 187-212.
15. Dumitriu S. Polysaccharides as biomaterials. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 1-45.
16. Zhang Y, Ni M, Zhang M, Ratner B. Calcium phosphate-chitosan composite scaffolds for bone tissue engineering. Tissue Eng. 2003 Apr;9(2):337-45.
17. Wang JW, Hon MH. Sugar-mediated chitosan/poly(ethylene glycol)-beta-dicalcium pyrophosphate composite: mechanical and microstructural properties. J Biomed Mater Res. 2003 Feb 1;64A(2):262-72.
18. Muzzarelli R, Biagini G, Pugnaloni A, Filippini O, Baldassarre V, Castaldini C, Rizzoli C. Reconstruction of parodontal tissue with chitosan. Biomaterials. 1989 Nov;10(9):598-603.
19. Zhang Y, Zhang M. Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. J Biomed Mater Res. 2001 Jun 5; 55(3): 304-12.

20. Ohgushi H, Caplan AI. Stem cell technology and bioceramics: from cell to gene engineering. J Biomed Mater Res. 1999;48(6):913-27.
21. Demers CN, Tabrizian M, Petit A, Hamdy RC, Yahia L. Effect of experimental parameters on the *in vitro* release kinetics of transforming growth factor beta1 from coral particles. J Biomed Mater Res. 2002 Mar 5;59(3):403-10.
22. Fricain JC, Roudier M, Rouais F, Basse-Cathalinat B, Dupuy B. Influence of the structure of three corals on their resorption kinetics. J Periodontal Res. 1996 Oct;31(7):463-9.
23. Irigaray JL, Oudadesse H, Blondiaux G. Quantitative study of the coral transformations '*in vivo*' by several physical analytical methods. Biomaterials. 1990 Jul;11:73-4.
24. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. Nat Biotechnol. 2000 Sep;18(9):959-63.
25. Shors EC. Coralline bone graft substitutes. Orthop Clin North Am. 1999 Oct;30(4):599-613.
26. Roux FX, Brasnu D, Loty B, George B, Guillemin G. Madreporic coral: a new bone graft substitute for cranial surgery. J Neurosurg. 1988 Oct;69(4):510-3.
27. Lucas A, Gaudé J, Carel C, Michel J.-F, Cathelineau G. A synthetic aragonite-based ceramic as a bone graft substitute and substrate for antibiotics. International Journal of Inorganic Materials. 2001 Jan; 3(1): 87-94.
28. Vuola J, Bohling T, Kinnunen J, Hirvensalo E, Asko-Seljavaara S. Natural coral as bone-defect-filling material. J Biomed Mater Res. 2000 Jul;51(1):117-22.
29. Roudier M, Bouchon C, Rouvillain JL, Amedee J, Bareille R, Rouais F, Fricain JC, Dupuy B, Kien P, Jeandot R, Basse-Cathalinat B. The resorption of bone-implanted corals varies with porosity but also with the host reaction. J Biomed Mater Res. 1995 Aug;29(8):909-15.
30. Doherty MJ, Schlag G, Schwarz N, Mollan RA, Nolan PC, Wilson DJ. Biocompatibility of xenogeneic bone, commercially available coral, a bioceramic and tissue sealant for human osteoblasts. Biomaterials. 1994 Jun;15(8):601-8.

31. Begley CT, Doherty MJ, Mollan RA, Wilson DJ. Comparative study of the osteoinductive properties of bioceramic, coral and processed bone graft substitutes. Biomaterials. 1995 Oct;16(15):1181-5.
32. Guillemin G, Meunier A, Dallant P, Christel P, Pouliquen JC, Sedel L. Comparison of coral resorption and bone apposition with two natural corals of different porosities. J Biomed Mater Res. 1989 Jul;23(7):765-79.
33. Patat JL, Guillemin G. Natural coral used as a replacement biomaterial in bone grafts. Ann Chir Plast Esthet. 1989;34(3):221-5.
34. Fricain JC, Alouf J, Bareille R, Rouais F, Rouvillain JL. Cytocompatibility study of organic matrix extracted from Caribbean coral porites astroides. Biomaterials. 2002 Feb;23(3):673-9.
35. Patel A, Honnart F, Guillemin G, Patat JL. Use of madreporaria coral skeletal fragments in orthopedic and reconstructive surgery: experimental studies and human clinical application. Chirurgie. 1980;106(3):199-205.
36. Pouliquen JC, Noat M, Verneret C, Guillemin G, Patat JL. Coral substituted for bone grafting in posterior vertebral arthrodesis in children. Initial results. Rev Chir Orthop Reparatrice Appar Mot. 1989;75(6):360-9.
37. Roux FX, Brasnu D, Menard M, Devaux B, Nohra G, Loty B. Madreporic coral for cranial base reconstruction. 8 years experience. Acta Neurochir (Wien). 1995;133(3-4):201-5.
38. Gravel M, Vago R, Tabrizian M. Use of Natural Coralline Biomaterials as reinforcing and forming agent for developing novel hybrid biomatrices: Microarchitectural and mechanical studies. Tissue Engineering. In press.
39. Gomi K, Davies JE. Guided bone tissue elaboration by osteogenic cells *in vitro*. J Biomed Mater Res. 1993 Apr;27(4):429-31.
40. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials. 1996 Jan;17(2):137-46.
41. Baksh D. Design strategies for 3-dimensional *in vitro* bone growth in tissue-engineering scaffolds. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 488-495.

42. Yaszemski MJ, Oldham JB, Lu L, Currier BL. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 541-547.
43. Muzzarelli C, Muzzarelli RA. Natural and artificial chitosan-inorganic composites. J Inorg Biochem. 2002 Nov 11;92(2):89-94.
44. Gottlib-Abramovitch. L, Geresh S and Vago R. Biofabricated marine hydrozoan: a bioactive crystalline material that promotes ossification of mesenchymal stem cells. Tissue Engineering. In press.
45. Sautier JM, Nefussi JR, Boulekbache H, Forest N. *In vitro* bone formation on coral granules. *In Vitro* Cell Dev Biol. 1990 Nov;26(11):1079-85.
46. Baxter LC, Frauchiger V, Textor M, ap Gwynn I, Richards RG. Fibroblast and osteoblast adhesion and morphology on calcium phosphate surfaces. Eur Cell Mater. 2002 Sep 30;4:1-17. Print 2002 Sep 30.
47. Sikavitsas VI, Temenoff JS, Mikos AG. Biomaterials and bone mechanotransduction. Biomaterials. 2001 Oct; 22(19): 2581-93.
48. Dahan D, Vago R, Golan Y. Skeletal architecture and microstructure of the reef building coral Fungia simplex. Mat. Sci. Eng. C. 2003; 23: 473-77.
49. Birk ZR, Gottlib-Aramovitch L, Margalit I, Aviv M, Forti E, Geresh S, Vago R. Conversion of adipogenic to osteogenic phenotype using crystalline porous biomatrices of marine origin. Tissue Engineering. In press.
50. Boyan BD, Schwartz Z. Modulation of osteogenesis via implant surface design. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 232-238.
51. Tisdel CL, Goldberg VM, Parr JA, Bensusan JS, Staikoff LS, Stevenson S. The influence of a hydroxyapatite and tricalcium-phosphate coating on bone growth into titanium fiber-metal implants. J Bone Joint Surg Am. 1994 Feb;76(2):159-71.
52. Burr DB, Mori S, Boyd RD, Sun TC, Blaha JD, Lane L, Parr J. Histomorphometric assessment of the mechanisms for rapid ingrowth of bone to HA/TCP coated implants. J Biomed Mater Res. 1993 May;27(5):645-53.
53. Frame MC, Fincham VJ, Carragher NO, Wyke JA. v-Src's hold over actin and cell adhesions. Nat Rev Mol Cell Biol. 2002 Apr;3(4):233-45.

54. Hunter A, Archer CW, Walker PS, Blunn GW. Attachment and proliferation of osteoblasts and fibroblasts on biomaterials for orthopaedic use. Biomaterials. 1995 Mar;16(4):287-95.

55. Trylovich DJ, Cobb CM, Pippin DJ, Spencer P, Killoy WJ. The effects of the Nd:YAG laser on *in vitro* fibroblast attachment to endotoxin-treated root surfaces. J Periodontol. 1992 Jul;63(7):626-32.

56. Rothamel D, Schwarz F, Sculean A, Herten M, Scherbaum W, Becker J. Biocompatibility of various collagen membranes in cultures of human PDL fibroblasts and human osteoblast-like cells. Clin Oral Implants Res. 2004 Aug;15(4):443-9.

57. Aubin JE. Osteogenic cell differentiation. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 21-28.

58. Sodek J, Cheifetz S. Molecular regulation of osteogenesis. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 31-42.

59. Khan Y, Pratt LT, Laurencin CT. Bone Graft Substitute Materials. emedicine. 2004 mar 3.

60. Maniatopoulos C, Sodek J, Melcher AH. Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res. 1988 Nov;254(2):317-30.

61. Peter SJ, Liang CR, Kim DJ, Widmer MS, Mikos AG. Osteoblastic phenotype of rat marrow stromal cells cultured in the presence of dexamethasone, beta-glycerolphosphate, and L-ascorbic acid. J Cell Biochem. 1998 Oct 1;71(1):55-62.

CHAPTER 6: CONCLUSION AND SUMMARY

The purpose of this study was to develop novel 3D coralline:chitosan macroporous composite scaffolds for bone tissue engineering, in which natural coral skeletal material was simultaneously used as particulate reinforcing phase and gas-forming agent. This gas-forming technique was combined with thermally induced phase separation. Macroporous chitosan scaffolds incorporated with different ratios of coralline material (25, 50, and 75 wt%) were fabricated and studied in comparison with pure chitosan and pure coral scaffolds. The coralline:chitosan weight ratio parameter was studied for its effects on the physical properties of the scaffolds and on cell behaviour.

Reinforced porous structures of large pore sizes were achieved by increasing the coralline:chitosan weight ratio. A clear evidence of the effect of coralline content on the physical properties of the scaffolds has been demonstrated. It was observed that with higher coralline proportions, the mixture of the composite was getting denser and more viscous, increasing pore wall thickness, and decreasing porosity, which consequently significantly improved the mechanical strength of the scaffold. Also, the generation of CO_2 from the chemical reaction happening between the coral and the acidic solvent used for chitosan polymer led to the growth of large gas pores, which considerably increased the mean pore diameter observed up to approximately 400 μm, more suitable for *in vitro* cell culture. Moreover, the density of coralline particles on the surface increased with higher coralline ratios, changing considerably the smooth surface morphology of pure chitosan.

The results of the *in vitro* cell culture study were promising. The introduction of the coral particulate phase into the chitosan scaffolds seemed to have favourably modified the surface microtopography, chemistry, and scaffold macroporosity, hence influencing positively the patterns of cell adhesion and morphology, proliferation and differentiation.

Striking results were obtained from the cells cultured on the coralline scaffolds, which obtained total cell numbers and ALP activity remarkably higher than all the other scaffolds. Moreover, they were the only material on which the osteocalcin protein was release throughout the study and generally at a high level. Such excellent performance of natural coral skeletal further comfort the idea of the selection of this biomaterial as filler in the production of chitosan-based scaffolds in order to promote osteoblastic differentiation. Accordingly, coral's favourable properties seemed to have allowed an optimization of the overall performance of the coralline:chitosan composite scaffolds. The coral-chitosan composite scaffolds containing high coral ratios generally obtained significantly higher results than the chitosan scaffolds in terms of total cell number and osteocalcin protein expression. In addition, of the chitosan-based scaffolds, the cells cultured on the 75:25 coral:chitosan scaffolds obtained the highest peak of ALP activity observed. On the other hand, the pure chitosan scaffolds and 25:75 coral:chitosan scaffolds generally obtained the lowest results.

Such different performances of the materials might be attributed to the distinct degree of cell adhesion revealed by the cell morphology observed by SEM. These results tend to indicate that coralline scaffolds offered surface conditions allowing the fastest cell attachment and spreading, corresponding to highest degree of cell adhesion observed in the present study. Throughout the 4 weeks of cell culture, the cells grown on coralline surfaces exhibited systematically flattened morphology with maximum cell surface adhesion. In comparison, the cells grown on the chitosan scaffolds exhibited a rounded morphology, indicating poor attachment. The cell spreading seemed to be considerably delayed and the cells seemed to have low proliferation rate. Nevertheless, the coralline-chitosan composites seemed to perform with a better cell affinity and behaviour in comparison to chitosan matrices, by allowing formation of numerous pseudopodial and filopodial extensions and faster cell spreading.

These results tend to indicate that the scaffolds containing coralline material seemed to perform better than their pure chitosan counterparts. Their surface conditions showed

improved cell affinity and behaviour by allowing better and faster attachment of MSCs, and a higher proliferation rate.

Within the limits of the present *in vitro* study, the results tend to indicate that the kinetic and degree of cellular adhesion were proportional to the coralline content, therefore influencing favourably the proliferation rate of the cells and osteogenic differentiation. Therefore, while the evaluation of cell morphology, cell number, ALP activity, and osteocalcin release expression, showed distinct patterns and level of expression depending on the type of material on which the cells were cultured, no particular effect of cell culture medium supplementation with β-glycerophosphate and dexamethasone was generally observed.

In summary, this study has introduced a novel method of fabricating reinforced macroporous chitosan-based composite scaffold with improved mechanical and biological properties concomitant with large pores extending the upper pore size limit of 250 μm obtained with freeze-dried scaffolds. In these composites, the coral particulate phase was judiciously chosen to act simultaneously as gas-forming and reinforcing agent. Such combination of coral biomaterial as gas-forming agent and a freeze-drying technique resulted in a simple, cost-effective, and rapid way of matrix production, avoiding the use of organic solvents or high temperatures. With the control over the scaffold parameters such as porosity, pore size, and mechanical properties, coralline:chitosan composite scaffolds have demonstrated an excellent potential as biomatrices for tissue engineering, having many advantages over the two individual homogeneous materials. Taken together, these results suggest that although the five groups of scaffolds support the development of a mature osteoblast phenotype, coralline scaffolds clearly have the best cellular adhesion, stimulate cell proliferation and enhance osteogenic differentiation, and that it is therefore an excellent material to be use as filler in the production of composite scaffolds. The results also suggest that coralline:chitosan composite scaffolds, especially with high coralline ratios, may lead to enhanced cell proliferation and expression of osteogenic markers, in comparison to chitosan scaffolds. In addition, when comparing to coralline scaffolds, the composite scaffolds, especially the 75:25 coral:chitosan wt%, have

significantly higher porosity and pore size; they are therefore less susceptible to cause, *in vitro,* cell-bridging and pore occlusion problems, while maintaining adequate mechanical properties and allowing potentially higher cell load. The coralline:chitosan composite scaffolds may be a definite optimization of the *in vitro* stage, and may contribute to the success of the tissue engineering strategy.

CHAPTER 7: Suggestions for future research

Adding larger amount of coralline material into the chitosan scaffolds could be performed along with further physical and biological characterisation on the composites, including studies on scaffold degradation, bioactivity, and *in vivo* implantation.

1. Adding larger amount of coralline material into the chitosan scaffolds.

In this study, three coralline:chitosan composites containing different ratios of coralline material (25, 50, and 75 wt%) were studied. The results suggested that the composite scaffolds with 75 wt% coralline ratio exhibited particularly better physical and biological properties in comparison with the pure chitosan scaffolds, but was still far from the biological performance of the coralline scaffolds. Therefore, in order to further enhance the physical properties and bring the biological performance closer to the ones obtained with coralline scaffolds, larger amount of coral hard particulate phase could be added into the scaffolds. Since at the beginning of this thesis, scaffolds containing 92 wt% of coral have been developed, and obtained mechanical properties indicating excellent structural integrity, performing extensive physical and biological studies could be useful to ascertain these expectations.

2. Evaluation of the in vitro degradation of the composite scaffolds could be performed.

The long-term performance of a cell-seeded scaffold is strongly affected by its degradation kinetic, by influencing potentially the cell behaviour such as growth and tissue regeneration [1]. Moreover, because the scaffold will start losing its mechanical strength as it degrades, its degradation and resorption kinetics should match tissue formation *in vitro* and/or *in vivo* to preserve its original physical and mechanical properties [2,3]. Scaffolds surface/volume ratio and pore wall thickness are some of the many physical parameters that have been identify to have an effect on the *in vitro* and *in vivo* degradation [1,4]. In this study, a clear evidence of the effect of coral content on various physical properties of the scaffolds, including the porosity, pore wall thickness,

pore size, and surface texture has been demonstrated. In addition, studies performed on hydroxyapatite-chitin matrixes composed with various hydroxyapatite fractions suggested that the hydroxyapatite particulate phase of the chitin matrix enhance the degradation rate of the scaffolds [5]. Therefore, the composite scaffolds containing different coralline weight ratios are expected to have different degradation rate. In order to improve our understanding and elucidate the effect of the coralline weight ratio on the degradation kinetic, studying the degradation could be assessed through gravimetry, mechanical testing, SEM, and micro-CT over time in a similar way as Behravesh *et al.*

3. Scaffold bioactivity testing.

Bioactivity refers to the ability of a material to form a biologically active carbonated hydroxyapatite layer to which bone forms directly on the surface and chemically binds to it [6,7,8]. The bioactive potential of scaffolding materials is thus of special interest since they can form a continuous interface with surrounding bone tissue [6]. The bioactivity of a scaffold can be determined by the formation of an apatite layer on the scaffolds surfaces after incubation in simulated body fluid [9]. A study performed by Zhang *et al.* suggested that an apatite layer was form on chitosan/β-tricalcium phosphate composite scaffold surfaces, while no layer was observed on pure chitosan scaffolds, or on the chitosan/ calcium phosphate invert glass. In order to elucidate if the incorporation of the coral particulate phase into the chitosan polymer would enhance the bioactive potential, the evaluation of the composite scaffolds' bioactivity would be interesting and may help us to better understand the different cell-material interaction observed on these composites.

4. In vivo implantation of coral-chitosan composite scaffolds.

In this study, the composite scaffolds containing different coral ratios exhibit different physical properties that may improve at different degrees their use in both *in vivo* and *in vitro* tissue-engineering applications [10]. The use of *in vitro* cellular testing usually complements *in vivo* animal testing. *In vitro* model system, however, does not replicate *in vivo* system, because of the numerous factors that would need to be considerate [11]. Therefore, performing *in vivo* tests, subcutaneous and non-load bearing area implantation,

of coralline:chitosan composite scaffolds would bring a greater understanding of the host tissue reaction, and bone integration [12].

REFERENCES

1. Lu L, Peter SJ, Lyman MD, Lai HL, Leite SM, Tamada JA, Vacanti JP, Langer R, Mikos AG. *In vitro* degradation of porous poly(L-lactic acid) foams. Biomaterials. 2000 Aug;21(15):1595-605.
2. Temenoff JS, Lu L, Mikos AG. Bone-tissue engineering using synthetic biodegradable polymer scaffolds. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp.454-459
3. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues-state of the art and future perspectives. J Biomater Sci Polym Ed. 2001;12(1):107-24.
4. Behravesh E, Timmer MD, Lemoine JJ, Liebschner MA, Mikos AG. Evaluation of the *in vitro* degradation of macroporous hydrogels using gravimetry, confined compression testing, and microcomputed tomography. Biomacromolecules. 2002 Nov-Dec;3(6):1263-70.
5. Ge Z, Baguenard S, Lim LY, Wee A, Khor E. Hydroxyapatite-chitin materials as potential tissue engineered bone substitutes. Biomaterials. 2004 Mar;25(6):1049-58.
6. Laurencin CT, Lu H.H. Polymer-Ceramic Composites for Bone-Tissue Engineering. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 462-468.
7. Ohgushi H, Caplan AI. Stem cell technology and bioceramics: from cell to gene engineering. J Biomed Mater Res. 1999;48(6):913-27.
8. Hench LL. Ceramics, Glasses, and Glass-Ceramics. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 73-83.

9. Zhang Y, Zhang M. Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. J Biomed Mater Res. 2001 Jun 5; 55(3): 304-12.
10. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials. 1996 Jan;17(2):137-46.
11. Baxter LC, Frauchiger V, Textor M, ap Gwynn I, Richards RG. Fibroblast and osteoblast adhesion and morphology on calcium phosphate surfaces. Eur Cell Mater. 2002 Sep 30;4:1-17. Print 2002 Sep 30.
12. Ma PX, Zhang R, Xiao G, Franceschi R. Engineering new bone tissue *in vitro* on highly porous poly(alpha-hydroxyl acids)/hydroxyapatite composite scaffolds. J Biomed Mater Res. 2001 Feb;54(2):284-93.

APPENDIX I: Manuscript I, in press.

Use of Natural Coralline Biomaterials as reinforcing and gas-forming agent for developing novel hybrid biomatrices: Microarchitectural and mechanical studies

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1. ABSTRACT

This paper introduces the first attempt of fabricating 3D macroporous composites of chitosan and natural coralline material having pore sizes of 300-400 μm exceeding the upper pore size limit of 250 μm obtained with freeze-dried chitosan-based scaffolds. Natural coral particulates sizing less than 20 μm, which is mainly composed of calcium carbonate ($CaCO_3$), was simultaneously used as reinforcing phase and gas-forming agent to obtain a structure with large pores and improved mechanical and biological properties. The reaction between the coralline material and the acidic chitosan polymer solvent, which produced carbon dioxide (CO_2), was rapidly stopped by the subsequent thermally induced phase separation step, leaving coralline particulates in the polymeric structure. Scaffolds containing five different proportions of coralline material (0, 25, 50, 75, and 100 wt%) were investigated. The coralline:chitosan weight ratio was studied for its effects on the physical properties of the scaffolds. The relation between scaffold microarchitecture and mechanical properties was assessed with scanning electron microscope (SEM) along with microcomputed tomography (micro-CT) imaging and compression testing. The scaffolds were used in bone marrow cell culturing experiments to assess the effect of composition on cell behaviour through cell-material interaction and morphological observation by SEM. Higher coralline concentration increased the pore wall thickness and favoured large pore formation. Varying the coralline particulate to chitosan polymer ratio from 0 to 75 wt% increased the average pore size from 80 μm to 400 μm while the porosity decreased from 91% to 78%. The compressive modulus was improved proportionally with the coralline content, and the 75 wt% composites had a significantly higher modulus than other chitosan-based scaffold groups. More cells were observed on scaffolds with higher coralline content. The cell culture experiments indicated that the scaffolds containing coralline material might have a high cell affinity, since it allowed fast cell attachment and spreading.

Keywords: Bone engineering, chitosan-based scaffolds, natural coral particulates, reinforcing and gas-forming agent, microstructure, porosity, mechanical properties, mesenchymal cell-material attachment and spreading.

2. INTRODUCTION

The most common strategy for engineering bone tissue is to use a hybrid biomaterial, formed from a combination of culture-expanded osteogenic cells seeded onto an appropriate temporary scaffold, which mimics the natural extracellular matrix [1-4]. The scaffold then gradually degrades, and is eventually replaced by the newly formed tissues. Such scaffold must satisfy a number of demanding requirements, namely biocompatibility, porosity, mechanical properties and adequate biodegradation rate [5]. Since no single material currently available provides all these essential features that an ideal scaffold requires, a logical approach is to design a composite that combines the favourable properties of each component while minimizing their shortcomings when used as homogenous materials [6-9].

To obtain such a composite, many researchers have recently proposed composite scaffolds of bioceramic particulates, such as calcium phosphate in the form of hydroxyapatite or β–tricalcium phosphate, incorporated into biodegradable polymers including chitosan to fabricate macroporous composite scaffolds with reinforced matrices and improved bioactivity [3,10-14]. Although these composite scaffolds have improved properties, it seems that none of them have achieved the ultimate goal of bone tissue engineering to create a device that has the capacity to replace autologous cancellous bone for the management of bony defect [15].

In this work, chitosan composite scaffolds, reinforced with natural coralline material are proposed as scaffold for bone engineering. Chitosan is one of the most promising polysaccharides for engineering numerous tissue systems, including bone tissue [16,17]. Made of glucosamine and *N*-acetyl-D-glucosamine groups [18], a molecular structure analogous to glycosaminoglycans, and having a cationic nature in physiological pH, chitosan exhibits multiple biological activities [16,19,20]. It is biocompatible, biodegradable, bioresorbable and has a hydrophilic surface, which facilitates cell adhesion, proliferation, and differentiation [11,20,21,22]. It has mitogenic activity on several different types of cells including osteoblasts, and has been reported to contribute to the differentiation of osteoprogenitor cells and to enhance and facilitate bone formation

[20,23-25]. Chitosan has the ability to be easily processed into porous structures [16]. The thermally induced phase separation (TIPS) technique is the most commonly mentioned approach to generate porous scaffold from chitosan [26]. This technique has also been called "freeze-drying" when the polymer solvent is an aqueous solution [27]. Porous structures can be easily produced with control over mean pore diameter by varying the freezing conditions and the chitosan concentration [17].

Although chitosan has many interesting properties, it lacks bioactivity and it is mechanically weak [14]. This is why many researches have undertaken to make chitosan-based composites. The addition of coralline derivatives is one of these approaches, since natural coralline has been widely used as a replacement biomaterial for bone grafts in different bone defects [2,28]. It possesses very important qualities for bone substitution due to its resorbability, osteoconductivity, bioactivity [2,29], adequate initial mechanical properties [2,28,30,31] and its good biocompatibility [2,32-34].

Calcium carbonate ($CaCO_3$) is the inorganic constituent of the skeletal material of natural coral [28] which reacts easily with acids and generates CO_2. This chemical property of coral exoskeleton is explored in the present study. By incorporating coralline particles into the chitosan polymer, they are not only used as particulate reinforcing phase but also as gas-forming agent. Our hypothesis was that the incorporation of coralline powders into chitosan polymers will result in a macroporous composite scaffolds with (i) enhanced mechanical properties due in part to the toughness and plasticity of the chitosan phase combined with the strength of the coralline phase [6,35]; (ii) increased osteogenic cells attachment due to changes in surface roughness and chemistry, and (iii) improved macroporosity [36] due to the generation of CO_2 in the acidic solution used as solvent for chitosan polymer. It must be noted that the freeze-drying processes currently used for chitosan and chitosan-based materials can only produce pore sizes up to 250 μm [17,27,37].

Chitosan-based hydrogel scaffolds containing three different ratios of coralline particles (25, 50, and 75 wt%) were prepared. The coralline:chitosan weight ratio parameter was studied for its effects on the physical properties of the scaffolds compared with two other groups of scaffolds made of their corresponding homogeneous material, namely coralline material and chitosan. The relationship between scaffold microarchitecture and mechanical properties was assessed using SEM, micro-CT imaging, and compression tests. In order to study the effect of the scaffold composition on cell behaviour, the scaffolds were cultured with mesenchymal cells (MSCs) and cell attachment capabilities were studied by SEM.

3. MATERIALS AND METHODS

3.1. Scaffold preparation

Chitosan solution with a concentration of 2 wt% was prepared by dissolving high viscosity chitosan (WA 1186 cPs, MW range from ~300–800 kDa, deacetylation degree 84.9 from Vanson HaloSource,) in a 0.2 M acetic acid. The solution was stirred at room temperature for 8 h and filtered through a fine cloth.

Coral skeletal material was obtained using small coral cores, from the species *Porites lutea,* harvested at the Seychelles archipelago. Exoskeleton blocs had an average pore size of 150 μm and an open porosity between 47-51%. They were soaked in NaOH 2N for 5 min, then treated with H_2O_2 30% for 10 min to remove trapped particles, debris and organic remnants. They were then broken in small pieces and shaped with sand paper to obtain flat squared coralline scaffolds measuring approximately 2x5x5 mm^3. The prepared coralline scaffolds were rinsed with distilled water and sterilized with an autoclave at 121°C for 40 min and oven-dried overnight at 80°C.

For chitosan-based scaffold preparation (Figure 1), the coralline particulate was obtained by milling solid coralline material for 8h in a rotating micromill. The particles were subsequently sieved through 20 μm U.S. standard sieve. Different proportion of coralline particulates (25, 50, and 75 wt%), sizing less than 20 μm, were added into the chitosan solution. The mixture was continuously stirred for 10 minutes to obtain homogeneous

slurry. The reaction between the coralline and the acidic chitosan solvent produced CO_2, and led to the formation of a viscous bubbly solution. Chitosan as reference materials and the chitosan composite mixtures were then poured into 96 multi-well polystyrene culture dishes. Samples were rapidly transferred into a freezer at –80°C overnight to solidify the solvent, stop the gas-forming reaction and induce a solid-liquid phase separation. The samples were then freeze-dried for 4 days at a temperature of
–52°C.

After lyophilisation, samples were neutralized by immersing them in absolute ethanol for 1h, followed by ethanol 70%/water (v/v) and 50% for 30 min each. The pH was adjusted with phosphate buffered saline (PBS) to 7.4 and scaffolds were rinsed with double-distilled water. The hydrated samples were frozen at –80°C, and cut with a razor blade into disks measuring approximately 2.5 mm in height and 5.5 mm in diameter. Finally, the scaffolds were freeze-dried and kept in a closed environment until use.

3.2. Microarchitectural analysis

The morphology of the scaffolds was examined with SEM (Hitachi field emission s-4700). The pore sizes were estimated from the SEM micrographs. Prior to SEM analysis, the samples were sputter-coated with gold-palladium under an argon atmosphere.

A Micro-CT system (SkyScan 1072, Belgium) was used to obtain 3D images from which samples' microarchitectural parameters such as porosity has been determined. The samples (n=4 for each coralline ratio group) were scanned using an energy of 39 kV and a current of 244 μA (9.5 watt power). A 180 degree scan was performed using a stepwise rotating angle of 0.45 degree with an integration time of 1782 msec for each image acquired with a solution of 4.56 μm. The cross-sections were reconstructed using Cone-Beam Reconstruction Software (SkyScan), having a distance 9.12 μm between each cross-section. Reconstructed array was shown as a half-tone image of cross section with linear conversion to 256-grades of grey inside selected density intervals which was kept the same for a sample group. Samples were analyzed with 3D Realistic Visualization software (Skyscan) to quantify the scaffolds architecture. The size of the region of interest

and the threshold value was the same for all the samples in order to be comparable and to cover their maximum volume. To distinguish pore from material, the segmentation method was used. The method consisted of selecting a threshold value in the 256-gray scale where each voxel is considered either material or pore.

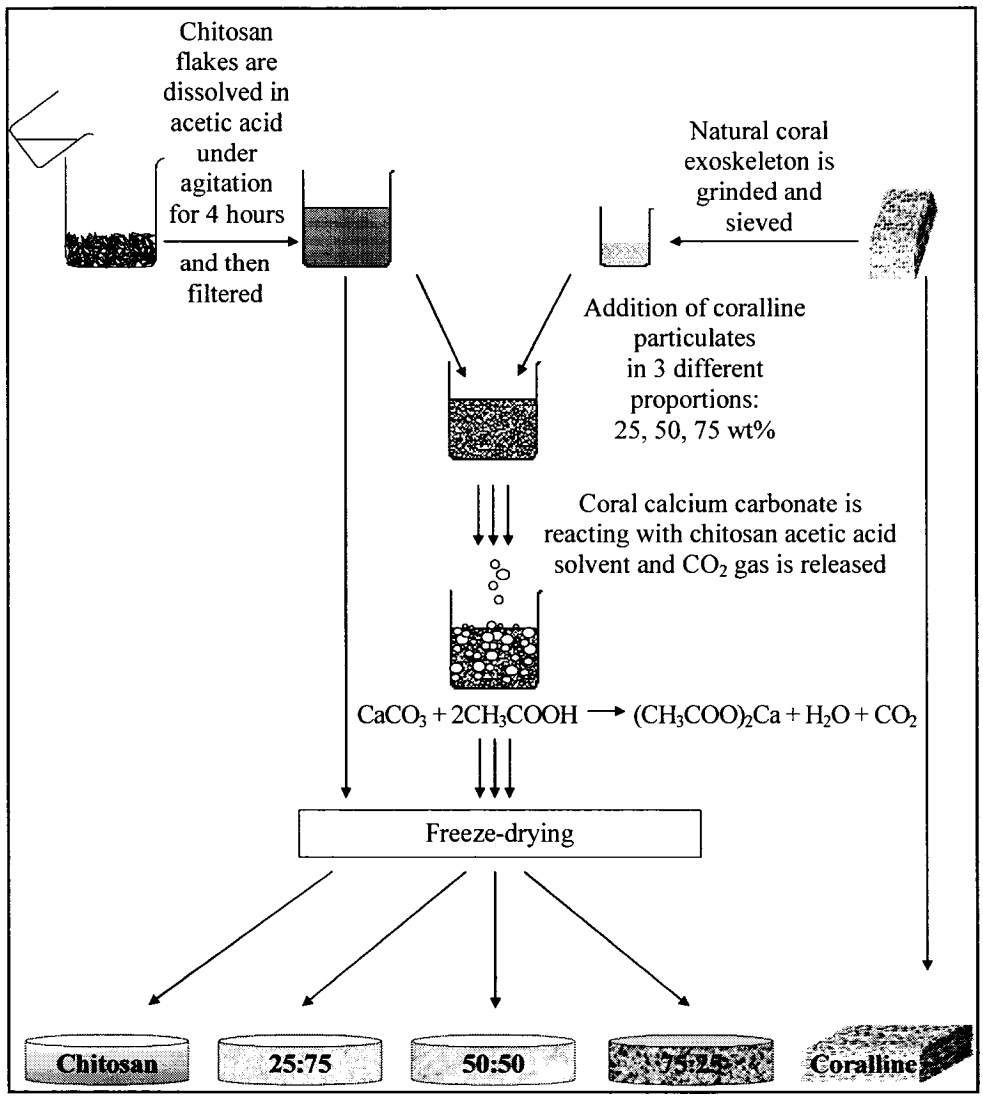

Figure 1. Schematic representation describing the production of the 5 groups of scaffolds. *The coralline particles, introduced in 3 different ratios into chitosan scaffolds, act both as a gas-forming and particulate-reinforcing agent: The reaction between the coral biomaterial and the acidic chitosan polymer solvent produced* CO_2 *which permeated through the chitosan to form pores. The reaction was rapidly stopped by freeze drying, leaving coral particulates reinforcing the polymeric porous structure.*

3.3. Compressive mechanical properties

The compressive equilibrium modulus was assessed from a stress relaxation function, using a Mach-1™ A400.25 mechanical tester (Biosyntech, Laval, PQ) reported to be accurate for the measurement of the mechanical properties of both biomaterials and biological tissues [38-44]. During the tests, the scaffolds were kept immersed in phosphate buffered saline solution (PBS). Using a load cell of 150g, the scaffolds were slowly subjected to 5 sequential step compressions of 15, 30, 45, 60%, 75% of strain, in uniaxial unconfined compression configuration. Four scaffolds per group were used. Under compression, the swollen scaffolds were thus allowed to expand in the radial direction and to expel the liquid through their porous media. Consequently, for each compression step, the load rapidly increased to the maximal strain and then relaxed to its equilibrium value (equilibrium stiffness). Equilibrium was defined as a change in the slope of the load relaxation curve less than 0.2 g/min, i.e. stabilized slope close to zero. A fixed relaxation time of 240 sec was used at each compression step to reach this equilibrium. The equilibrium modulus for each step was then determined from the ratio of the equilibrium stress minus the initial stress over the strain [45].

3.4. Cell culture

MSCs (mouse cell line CRL-12424 from ATCC, Manassa, VA, USA), were cultured for 5 passages in Dulbecco's modified Eagle medium (DMEM) medium (Gibco, Burlington, ON, Canada), supplemented with 4 mM-glutamine, 4.5 g/L glucose, and 25 mM HEPES buffer, 10% fetal bovine serum (Gibco), 10 U/ml penicillin G sodium, 10 μg/ml streptomycin and 25 μg/ml amphotericin B as Fungizone (Gibco), and 100 μg/ml L-ascorbic acid (Sigma-Aldrich, Oakville, ON, Canada).

A low-density cell seeding was assessed in order to avoid cell-cell contact and to achieve easier visualization of individual cell interaction with the scaffold material. In a 48 multi-wells plate, aliquots of 10 μl and 40 μl cell suspension were seeded onto the dry coralline and chitosan-based samples placed in wells, resulting in a seeding density of $5x10^5$ cells/cm^2. 150 μl of medium were added into each well. The scaffolds were incubated for 12 h to allow cells to attach to surface, and were then transferred into 35 mm tissue

culture polystyrene dishes. Media were changed every 2-3 days. The cell-material interactions were studied after 3, 7, 21 days with SEM (Hitachi field emission s-4700). Coralline and chitosan scaffolds were used as reference materials.

3.5. Cell morphology analysis by SEM

The morphology of cells was examined with SEM. The cell-seeded scaffolds were rinsed twice with PBS, fixed with 2.5% glutaraldehyde in 0.14M sodium cacodylate (pH 7.4) for 24h at 5°C and dehydrated by 30, 50, 70, 80, 90, and 100% ethanol for 10 min for each. Further substitution to amyl acetate was performed through four graded bath of amyl acetate: ethanol (25:75), followed by (50:50), (75:25), and (100%). Prior to SEM observation, samples were critically point dried and covered with a thin layer of gold-palladium through sputtering under an argon atmosphere.

3.6. Statistical Analysis

Multiple samples were collected for porosity measurements as well as for mechanical characterization. Data were reported as means ± standard deviations. One-way analysis of variance (ANOVA) with Scheffe's multiple comparison tests was used to assess the statistical significance between the porosity of the groups. The same ANOVA statistical analysis using Tukey's multiple comparison tests was performed to evaluate the difference within each compression step.

4. RESULTS

4.1. Analysis of compressive mechanical properties

The measurement of compressive equilibrium modulus showed that the chitosan-based scaffolds were reinforced proportionally by the addition of coralline powder phase (Figure 2). Although this trend was not supported by a significant difference for the 0, 25, and 50 % coralline weight ratios, the 75 wt% composites modulus was significantly higher ($p<0.001$) than all the other scaffold groups as soon as 30% strain was applied.

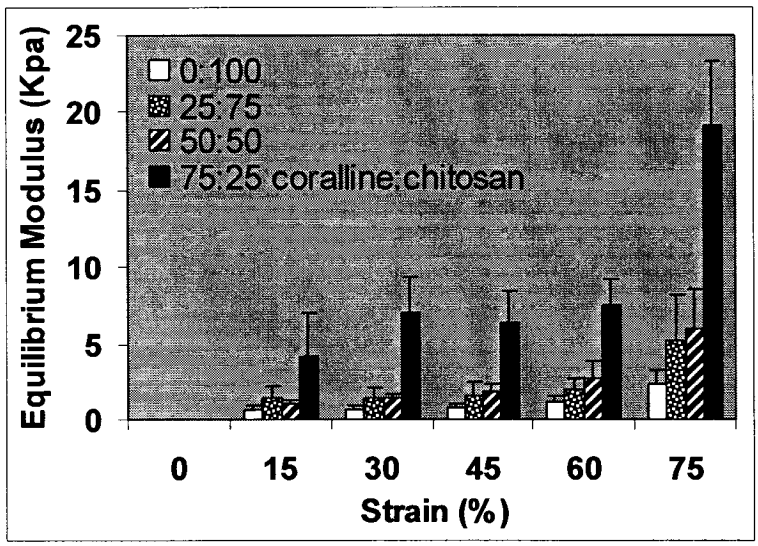

Figure 2. The compressive equilibrium modulus of macroporous 0:100, 25:75, 50:50, and 75:25 coralline:chitosan composites. Equilibrium mechanical testing was conducted between 0% and 75% strain in 5 steps of 15% strain increments. Error bars represent means standard deviations for n = 4.

4.2. Analysis of scaffold morphology

The presence of the coralline particles in the polymeric scaffolds considerably changed the surface morphology (Figure 3 and 4). The concentration of rough coral particles observed on the composite scaffold walls and on the surfaces increased with the coralline ratio. The distribution of coralline particles was homogeneous and no trend to agglomeration has been observed.

As indicated in Figure 5, an increase of coralline content slightly and linearly decreased porosities allowing good control over the porosity of the chitosan-based scaffold. Significant differences were found only between the 0%-50 w% and between the 25-75 w% groups with $p<0.1$. Increasing the coralline content from 0% to 75 wt% decreased significantly ($p<0.01$) the porosity from 91% (± 0.44) to 77.85% (± 6.96).

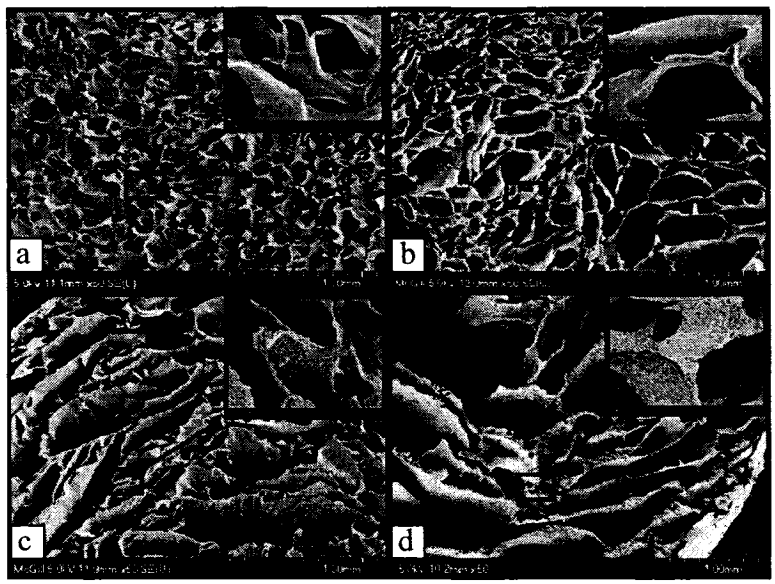

Figure 3. Cross-sectional SEM micrographs of chitosan-based scaffolds containing varying amount of coralline material. *Representation (x50) of* ***a.*** *0:100;* ***b.*** *25:75;* ***c.*** *50:50;* ***d.*** *75:25 coralline:chitosan weight ratios. The upper insets illustrate an x500 high magnification of corresponding scaffold.*

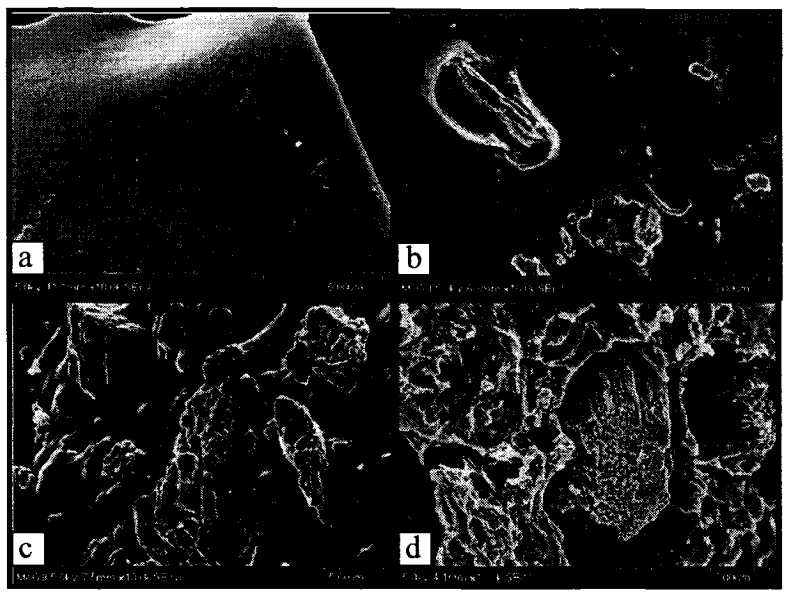

Figure 4. SEM micrographs of chitosan-based scaffold surfaces containing varying amount of coralline material. *Representation (x5k) of* ***a.*** *0:100* ***b.*** *25:75,* ***c.*** *50:50, and* ***d.*** *75:25 coralline:chitosan weight ratios.*

In addition to above-mentioned structural characteristics, coralline biomaterial concomitantly created larger pores into composite scaffolds. The evolving gas permeated through the chitosan and led to the growth of pores. The pore size as revealed by SEM images clearly increased with coralline content from 80 μm (average size for the chitosan scaffold) up to a mean of approximately 400 μm for the 75:25 coralline:chitosan wt% composite scaffolds, sometimes reaching a maximum pore sizes of about 1000 μm (Figures 3 and 6). The more coralline content there was, the more evolving gas permeated through the chitosan, leading to the growth of larger gas pores. The coralline:chitosan composites resulted in an open pore macrostructure on the surfaces, as demonstrated in both SEM micrographs (Figure 3) and the micro-CT reconstruction image (Figure 6).

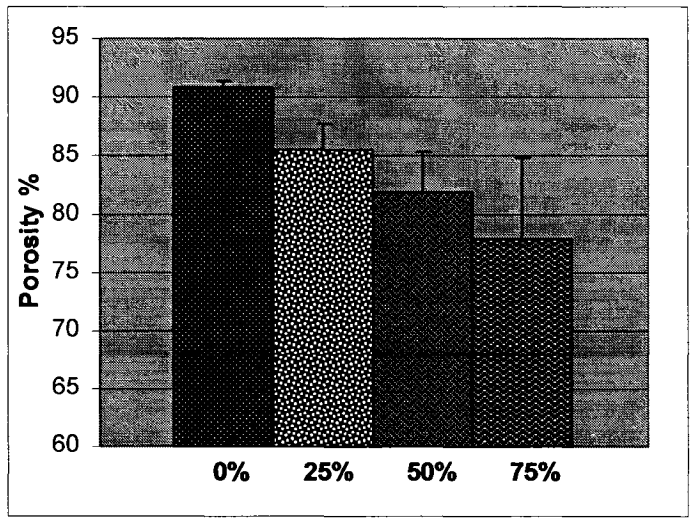

Figure 5. Micro-CT analyses showing different porosities for the chitosan-based scaffolds containing 0, 25, 50, and 75 wt% of coralline material. *Error bars represent means ± standard deviations for n = 6. (n = 4 for 0% study group) (0%-50% p<0.1, 25%-75% p<0.1, 0%-75% p<0.01).*

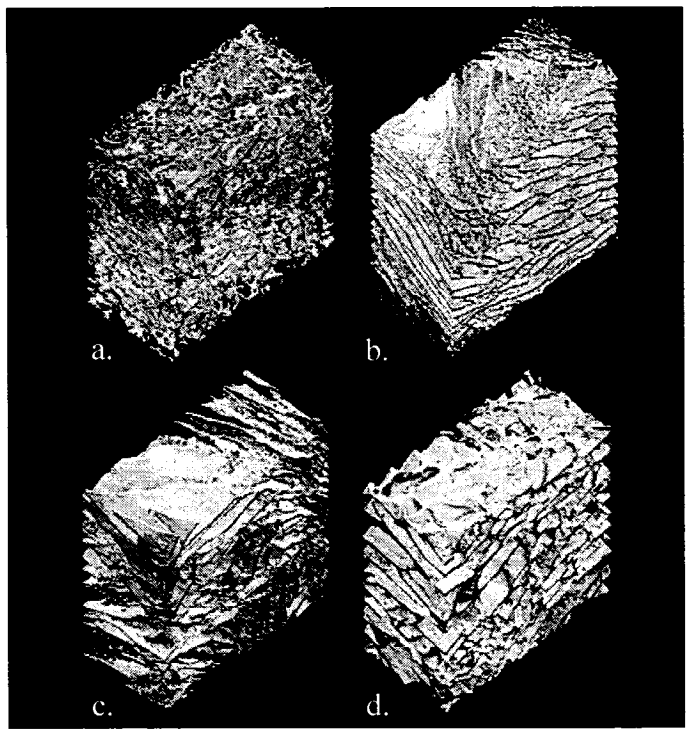

Figure 6. Representative micro-CT images of the macroporous scaffolds. ***a.*** *0:100;* ***b.*** *25:75;* ***c.*** *50:50;* ***d.*** *75:25 coralline:chitosan composites.*

The pore uniformity of the composite microstructures was affected by the presence of coralline material. The pure chitosan scaffolds had a homogeneous three-dimensional porous structure with small pore size range regular in shape. While adding 25 wt% of coral particulates did not seem to affect significantly the uniformity of the pore morphology, adding 50 or 75 wt% of coralline material into chitosan scaffolds resulted in structures with irregular pore morphology and larger pore size.

4.3. SEM analysis of cell attachment and spreading onto and into scaffold

The relationship between the scaffolds properties and their biological performance was investigated through cell adhesion and morphological studies. Throughout the three weeks of cell culture, the cells grown on coralline surfaces assumed distinct morphology compared to the cells grown on the chitosan-based scaffolds. During the entire study, every single MSC grown on the coralline scaffolds systematically exhibited highly flattened star shape morphology with many projections. As soon as one week after the

seeding, a great number of cells were observed on those scaffolds starting to form a thin cell layer on the surface (Figure 7a). In contrast, the great majority of cells grown on the chitosan scaffolds, remained rounded for more than one week. Figure 7c shows such typical round cells found on chitosan scaffolds for which the cell spreading was considerably delayed. After one week of culture, few cells randomly distributed could be observed. Although most of cells were also round after three days of culture on the coralline:chitosan composite scaffolds, some spread cells having large and extended cell bodies with signs of activity such as microvilli and fibre-like processes were also found (Figure 7b).

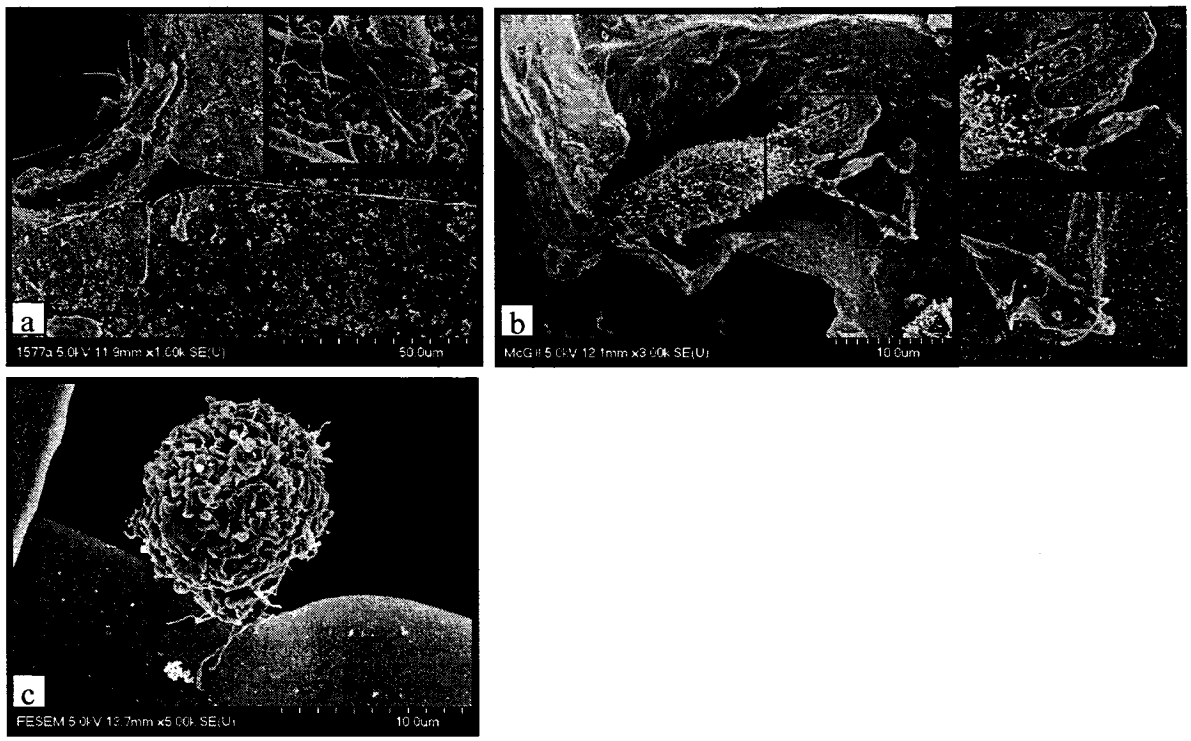

Figure 7. SEM micrographs showing distinct differences in morphology between cells cultured for three days on coralline and chitosan-based scaffolds. ***a.*** *Cells on coralline scaffolds exhibiting flattened star shape morphology with numerous microspikes and filopodia, sometimes 100 μm long (magnification x1k, inset x20k);* ***b.*** *Spread cells on 25:75 coralline:chitosan composite scaffolds developing focal attachment with coral particles, representative the common feature of cell-coralline:chitosan scaffold interactions (magnification x3k, the upper and lower insets are a 10k and 20k high-magnification of filopodia);* ***c.*** *Cells on chitosan surface exhibiting a rounded shape (magnification x5k).*

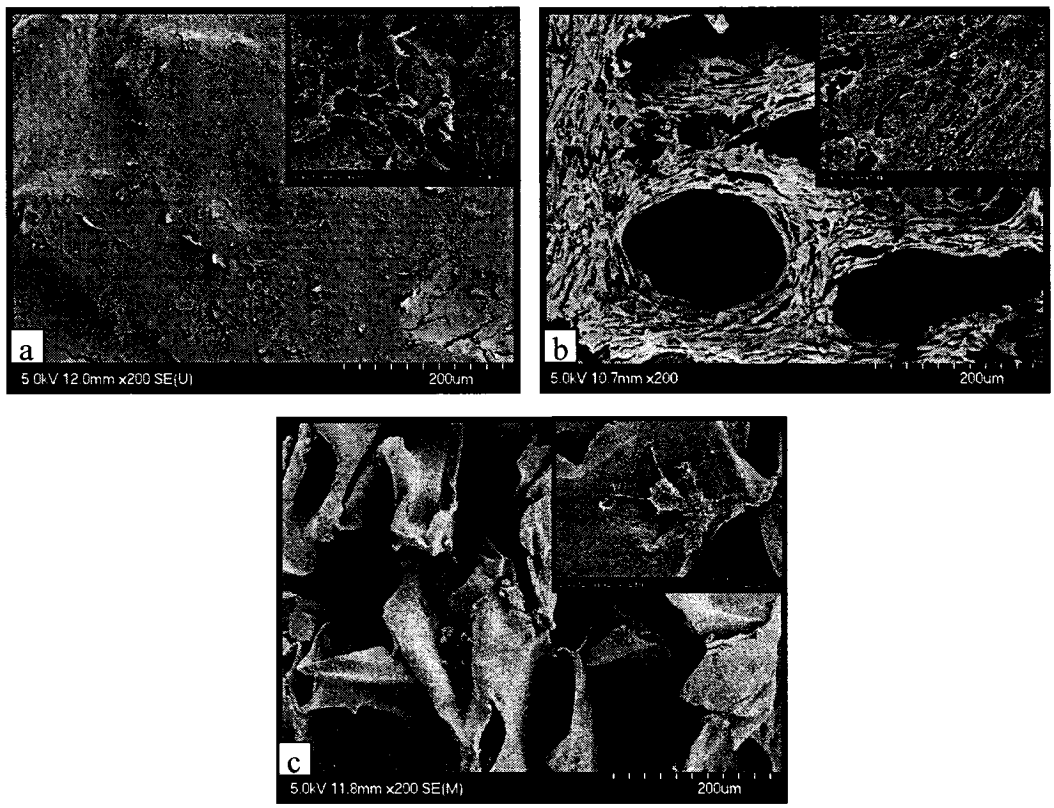

Figure 8. SEM micrographs of samples cultured with mice mesenchymal stem cells at 3 weeks. *(Magnification x200, insets x2k).* ***a.*** *Cell sheet on the coralline scaffolds covering the entire porous network with evidence of complete pore occlusion in cell culture;* ***b.*** *Cells grown on the surface of 75:25 coralline:chitosan composite scaffolds without sign of pore occlusion;* ***c.*** *Cells on chitosan scaffolds characterized by mostly isolated cells a few cell aggregations.*

After three weeks of culture, the cells covering the surfaces were quite different among the scaffold groups and seemed to be influenced by the coralline ratio (Figure 8). On the coralline scaffolds, a thick layer of cell had been laid down over the entire porous network and caused complete pore occlusions (Figure 8a).

On the 50:50 and 75:25 wt% coralline:chitosan scaffolds, a cell layer was covering the surfaces without blocking the lager pores (Figure 8b). Interestingly, virtually all of spread cells were forming focal contacts with coralline particles. The chitosan scaffolds were

still characterized by the presence of mostly isolated cells along with few cell aggregations into the scaffold even though some signs of cell spreading and microvilli and fibre-like processes could be observed (Figure 8c).

5. DISCUSSION

Developing scaffolds for bone tissue engineering aim at enhancing mechanical properties while producing highly porous structures to promote cell delivery. This results in conflicting design requirements and goals [46,47]. To conciliate the physical and mechanical requirements, scaffold porosity should be at a maximum as long as the mechanical features are not compromised [48,49]. Although this is a generally accepted concept, the optimal magnitudes of the mechanical requirements of a temporary scaffold are not established [47]. We proposed here, the use of coralline particulate phase to act simultaneously as gas-forming and reinforcing agent to obtain a chitosan-based composite scaffold with improved mechanical and biological properties concomitant with large pores exceeding the upper pore size limit of 250 μm obtained with freeze-dried scaffolds. Choi *et al.* and Chow *et al.* also reported that synthetic $CaCO_3$ as gas-forming agent is highly effective and that the increase of its weight ratio increased the pore diameter and porosity [37,50]. But since a complete reaction occurred and no residual of reinforcing calcium salts was left in the resulting homogeneous material, the largest pore structure was associated with the lowest mechanical strength [37,50].

In the present work, unlike these two reports, the reaction between the coralline $CaCO_3$ and the acidic chitosan solution was rapidly stopped by freezing the samples in the subsequent thermally induced phase separation step (TIPS), leaving coralline particulates to reinforce the polymeric porous structure. As the coralline ratio was raised, the composite suspension became more viscous and consequently the pore wall thickness increased along with a decrease in porosity leading to scaffolds with greater mechanical strength. As a consequence, chitosan-based scaffolds were reinforced proportionally with the addition of coralline particulate phase (Figure 2), and the 75:25 coralline:chitosan composite scaffolds provided the largest pore structure with highest compressive properties.

These observations are consistent with those reported earlier with freeze-dried composite polymer scaffolds, where the composites have improved mechanical properties and decreased porosity over those of the pure polymer scaffolds [27,51-53]. However, contrarily to some other reports, in which the incorporation of particulate weight ratio beyond 50% caused a dramatic decrease of the mechanical strength [10,51], the structural integrity of the composite scaffolds developed in the current study were all reinforced. The homogenous distribution of particulate might contribute to the good integrity of the composite scaffolds in our case, since agglomerations of reinforcing phase have been reported to be potentially detrimental to the compressive properties of composite scaffolds [27].

In addition to above-mentioned structural characteristics, the novelty of this method is that coralline material as gas-forming agent into composite concomitantly created larger pores over both coralline and chitosan scaffolds. Chow *et al.* have reported similar results by obtaining a continuous pore structure from the bulk to the surface of the scaffolds with pore sizes ranging from 100 to 1000 μm depending on the amount of $CaCO_3$ added [37]. Furthermore, as shown by SEM analysis and micro-CT reconstruction imaging (Figures 3 and 6 respectively), all the coralline:chitosan composites resulted in an open pore macrostructure on the surfaces. Porogen particles, which are the initiators of gas formation, were dispersed into the polymer and caused the evolving gas to permeate from within the bulk to the surface of the material, leaving gas bubbles or pores. However freezing the scaffolds as the CO_2 was released, allowed the matrix porous structures to remain intact after the subsequent lyophilisation process and also contributed to prevent the surface and internal pore to collapse during dehydration process. Our method thus represents an advantage over the gas-foaming technique where the fast diffusion of the gas to the surface collapses the external pores leading to a nonporous surface [30,54].

When the 75:25 coralline:chitosan scaffolds are compared with reference materials, their higher mean porosity (77.85% versus 50%) comparing to coralline might potentially provide higher cell load and attachment enabling presumably faster healing process once implanted into the patient [14]. Conversely, their lower porosity in comparison to

chitosan scaffolds provides higher resistance to compression. This might be a suitable compromise since the improvement of scaffold macroporosity, microtopography and chemistry are parameters known to affect osteogenic cells *in vitro* [36,55] and tissue ingrowth capabilities [27,47].

The increased pore size obtained with the coralline:chitosan composite scaffolds has several advantages over the small pore size of chitosan or coralline scaffolds. We have shown that with an average pore size of 150 μm, complete pore occlusion could occur over the entire porous network of the coralline scaffolds after three weeks of cell culture (Figure 8a). Similar results indicated that pore occlusion by cells in biomatrices occurred when the pore size was less than 200 μm [36]. Pore occlusions prevent further cell and tissue ingrowth throughout the scaffold and may affect nutrients circulation and cell metabolites clearance from the scaffolds [36]. Our technique produced composites with an average pore of 400 μm which seems to be in the range of an optimal of pore size. Indeed, previous studies have shown that the nominal pore sizes between 300 to 500 μm [28,36,56] may reduce or obviate the problems of pore occlusion. It could provide curvatures with optimal compression and tension on osteoblast mechanoreceptors, stimulating migration, attachment and proliferation [57], and would allow three-dimensional tissue growth with an optimal bone ingrowth velocity [22,58].

Our SEM observations tend to indicate that the presence of coralline material influence favourably the patterns of cell adhesion/morphology and the biological responses to the different scaffolds. MSCs grown on the pure coralline scaffolds systematically exhibited highly flattened star shape morphology with many projections. As soon as one week of culture, a layer of cell was laid down over the entire porous network. In contrast, on the chitosan scaffolds, only few cells could be observed and the cell morphology remained rounded. The presence of rounded cell on chitosan samples, was in agreement with the previous *in vitro* study reported by Lahiji *et al.* who demonstrated that over 90% of the osteoblasts cultured for a period of seven days retained a rounded appearance on a chitosan-coated surface while greater than 90% of these cells grown on uncoated plastic coverslips assumed a spindle shaped, fusiform appearance [59].

These results are differing from several other studies suggesting that chitosan facilitates osteoprogenitor cell adhesion, contributes to their differentiation, and enhances bone formation [23,60.61]. We assume that the low cell density seeding used both by Lahiji *et al.* and in our study [62-65], might explain our results and the delay in the cell spreading on chitosan scaffolds. In fact, cells cultured on both chitosan-coated and uncoated surfaces were randomly distributed at low concentration to avoid cell overlapping and the formation nor formed clusters [59]. Similarly, a low cell-seeding density was performed in our study to allow easier visualization of individual cell interaction with the scaffold material. In both cases, intercellular contacts could not contribute to the cellular regulation.

Nevertheless, the addition of coralline material had a positive impact on the kinetic and degree of adhesiveness as well as on the numbers of focal attachments the cells formed with the substratum. In comparison to the chitosan scaffolds, much more spread cells were observed on the coralline:chitosan scaffolds after one week of culture, most of which were forming focal contacts with coralline particles. Cells forming the highest number of focal contacts have been reported to exhibit a well spread and flattened morphology whereas those with the least number of focal contacts assume a more rounded and less spread shape [66]. Flattened cells by well-defined attachment extensions and several lamellopodia like those observed on the coralline scaffolds, are known to be firmly attached onto surface and have a higher rate of proliferation than cells assuming a rounded morphology as a sign of poor attachment like those observed on the chitosan scaffolds [66-68].

Besides anchoring cells, focal contacts are of enormous importance in the control of cell phenotype, and thus potentially determine the success of the scaffolds [57,66]. By binding to the external environment via integrin receptors, focal contacts play an important role in relaying signals from the material substrates to the cytoskeleton and nucleus, which is affecting the cell shape, gene transcription, and expression of specific phenotypes [69]. The surface texture or microtopography as well as chemistry plays also an important role in the cellular response and adhesion [36,55,58,69,70]. Therefore the rougher topography

and different chemistry caused by the presence of coralline in the composite scaffolds have had a positive impact on the number of focal attachments. This represents a relevant feature of our approach since adhesion to substrate is the first step to cell viability, growth, spreading and differentiation [71].

6. CONCLUSION

By combining chitosan with different ratios of natural coralline material, which are simultaneously used as particulate reinforcing phase and gas-forming agent, followed by thermally induced phase separation, a family of reinforced macroporous scaffolds with large pore size was developed. Such production of macroporous structures whose integrity is not only maintained but significantly reinforced, suggests that chitosan and coral skeletal material present a great combination to feature an optimized scaffold. With the control over the scaffold parameters such as porosity, pore size, mechanical properties, and cellular affinity, our coralline:chitosan composite scaffolds have demonstrated very interesting structural characteristics justifying our judicious choice of natural coralline biomaterial as a typical particulate reinforcing phase as well as gas-forming agent. Our technique benefits from the chemical reaction between its components that generate CO_2 which contribute to the scaffold's pore morphology. The proposed method is simple, cost-effective, and avoids the use of organic solvents or high temperatures. It requires no additional components or chemicals compared to regular composite scaffolds of polymer and bioceramics that are freeze-dried. The results demonstrate that the kinetic and degree of cellular adhesion were proportional to the coralline content, and may in turn influence favourably the cellular proliferation and differentiation. Our composite scaffolds possess therefore many advantages, such as improved porosity and mechanical properties over coralline and chitosan scaffolds respectively, as well as optimal pore size and biological activities, not only over these two individual homogeneous materials but also comparing to many other scaffolds suggesting that new coralline:chitosan composites have a great potential as biomatrices for tissue engineering. Extensive *in vitro* experiments are currently ongoing to further investigate the biological response to these novel coralline:chitosan composite scaffolds and to prove the concept.

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8. REFERENCES

1. Yaszemski MJ, Oldham JB, Lu L, Currier BL. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 541-547.
2. Ohgushi H, Caplan AI. Stem cell technology and bioceramics: from cell to gene engineering. J Biomed Mater Res. 1999;48(6):913-27.
3. Zhao F, Yin Y, Lu WW, Leong JC, Zhang W, Zhang J, Zhang M, Yao K. Preparation and histological evaluation of biomimetic three-dimensional hydroxyapatite/chitosan-gelatin network composite scaffolds. Biomaterials. 2002 Aug;23(15):3227-34.
4. Risbud M. Tissue engineering: implications in the treatment of organ and tissue defects. Biogerontology. 2001;2(2):117-25.
5. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues-state of the art and future perspectives. J Biomater Sci Polym Ed. 2001;12(1):107-24.
6. Laurencin CT, Lu H.H. Polymer-Ceramic Composites for Bone-Tissue Engineering. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 462-468.
7. Gunatillake PA, Adhikari R. Biodegradable synthetic polymers for tissue engineering. Eur Cell Mater. 2003 May 20;5:1-16.

8. Khan SN, Tomin E, Lane JM. Clinical applications of bone graft substitutes. Orthop Clin North Am. 2000 Jul;31(3):389-98.
9. Wang M. Developing bioactive composite materials for tissue replacement. Biomaterials. 2003 Jun;24(13):2133-51.
10. Zhang Y, Zhang M. Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. J Biomed Mater Res. 2001 Jun 5; 55(3): 304-12.
11. Zhang Y, Ni M, Zhang M, Ratner B. Calcium phosphate-chitosan composite scaffolds for bone tissue engineering. Tissue Eng. 2003 Apr;9(2):337-45.
12. Zhang Y, Zhang M. Calcium phosphate/chitosan composite scaffolds for controlled *in vitro* antibiotic drug release. J Biomed Mater Res. 2002 Dec 5;62(3):378-86.
13. Ge Z, Baguenard S, Lim LY, Wee A, Khor E. Hydroxyapatite-chitin materials as potential tissue engineered bone substitutes. Biomaterials. 2004 Mar;25(6):1049-58.
14. Zhang Y, Zhang M. Three-dimensional macroporous calcium phosphate bioceramics with nested chitosan sponges for load-bearing bone implants. J Biomed Mater Res. 2002 Jul; 61(1): 1-8.
15. Linhart W, Peters F, Lehmann W, Schwarz K, Schilling AF, Amling M, Rueger JM, Epple M. Biologically and chemically optimized composites of carbonated apatite and polyglycolide as bone substitution materials. J Biomed Mater Res. 2001 Feb;54(2):162-71.
16. Matthew HW. Polymers for Tissue Engineering Scaffolds. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 170-186.
17. Madihally SV, Matthew HW. Porous chitosan scaffolds for tissue engineering. Biomaterials. 1999 Jun;20(12):1133-42.
18. Suh JK, Matthew HW. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials. 2000 Dec;21(24):2589-98.
19. Domard A, Domard M. Chitosan: Structure-properties relationship and biomedical applications. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 187-212.

20. Dumitriu S. Polysaccharides as biomaterials. In Polymeric Biomaterials, 2nd Edition, Revised and Expanded, S. Dumitriu, ed., Marcel Dekker, New York, 2001, pp. 1-45.
21. Wang JW, Hon MH. Sugar-mediated chitosan/poly(ethylene glycol)-beta-dicalcium pyrophosphate composite: mechanical and microstructural properties. J Biomed Mater Res. 2003 Feb 1;64A(2):262-72.
22. Muzzarelli R, Biagini G, Pugnaloni A, Filippini O, Baldassarre V, Castaldini C, Rizzoli C. Reconstruction of parodontal tissue with chitosan. Biomaterials. 1989 Nov;10(9):598-603.
23. Klokkevold PR, Vandemark L, Kenney EB, Bernard GW. Osteogenesis enhanced by chitosan (poly-N-acetyl glucosaminoglycan) *in vitro*. J Periodontol. 1996 Nov;67(11): 1170-5.
24. Cho BC, Park JW, Baik BS, Kwon IC, Kim IS. The role of hyaluronic acid, chitosan, and calcium sulfate and their combined effect on early bony consolidation in distraction osteogenesis of a canine model. J Craniofac Surg. 2002 Nov;13(6):783-93.
25. Kind GM, Bines SD, Staren ED, Templeton AJ, Economou SG. Chitosan: evaluation of a new hemostatic agent. Curr Surg. 1990 Jan-Feb;47(1):37-9.
26. Khor E, Lim LY. Implantable applications of chitin and chitosan. Biomaterials. 2003 Jun;24(13):2339-49.
27. Yin Y, Ye F, Cui J, Zhang F, Li X, Yao K. Preparation and characterization of macroporous chitosan-gelatin/beta-tricalcium phosphate composite scaffolds for bone tissue engineering. J Biomed Mater Res. 2003 Dec 1;67A(3):844-55.
28. Demers CN, Tabrizian M, Petit A, Hamdy RC, Yahia L. Effect of experimental parameters on the *in vitro* release kinetics of transforming growth factor beta1 from coral particles. J Biomed Mater Res. 2002 Mar 5;59(3):403-10.
29. Patat JL, Guillemin G. Natural coral used as a replacement biomaterial in bone grafts. Ann Chir Plast Esthet. 1989;34(3):221-5.
30. Irigaray JL, Oudadesse H, Blondiaux G. Quantitative study of the coral transformations '*in vivo*' by several physical analytical methods. Biomaterials. 1990 Jul;11:73-4.

31. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. Nat Biotechnol. 2000 Sep;18(9):959-63.
32. Roudier M, Bouchon C, Rouvillain JL, Amedee J, Bareille R, Rouais F, Fricain JC, Dupuy B, Kien P, Jeandot R, Basse-Cathalinat B. The resorption of bone-implanted corals varies with porosity but also with the host reaction. J Biomed Mater Res. 1995 Aug;29(8):909-15.
33. Begley CT, Doherty MJ, Mollan RA, Wilson DJ. Comparative study of the osteoinductive properties of bioceramic, coral and processed bone graft substitutes. Biomaterials. 1995 Oct;16(15):1181-5.
34. Guillemin G, Meunier A, Dallant P, Christel P, Pouliquen JC, Sedel L. Comparison of coral resorption and bone apposition with two natural corals of different porosities. J Biomed Mater Res. 1989 Jul;23(7):765-79.
35. Muzzarelli C, Muzzarelli RA. Natural and artificial chitosan-inorganic composites. J Inorg Biochem. 2002 Nov 11;92(2):89-94.
36. Baksh D. Design strategies for 3-dimensional *in vitro* bone growth in tissue-engineering scaffolds. In Bone Engineering. Davies JE, ed., em sqared incorporated, Toronto, 2000, pp. 488-495.
37. Chow KS, Khor E. Novel fabrication of open-pore chitin matrixes. Biomacromolecules. 2000 Spring;1(1):61-7.
38. Ravenelle F, Marchessault RH, Légaré A, Buschmann MD. Mechanical properties and structure of swollen crosskinked high amylose starch tablets. Carbohydrate Polymers. 2002 Jan; 47: 259-266.
39. Jurvelin JS, Buschmann MD, Hunziker EB. Optical and mechanical determination of Poisson's ratio of adult bovine humeral articular cartilage. J Biomech. 1997 Mar;30(3):235-41.
40. Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. The use of specific chondrocyte populations to modulate the properties of tissue-engineered cartilage. Orthop Res. 2003 Jan;21(1):132-8.

41. Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. Characterization of cartilagenous tissue formed on calcium polyphosphate substrates *in vitro*. J Biomed Mater Res. 2002 Dec 5;62(3):323-30.
42. Garon M, Legare A, Guardo R, Savard P, Buschmann MD. Streaming potentials maps are spatially resolved indicators of amplitude, frequency and ionic strength dependant responses of articular cartilage to load. J Biomech. 2002 Feb;35(2):207-16.
43. Legare A, Garon M, Guardo R, Savard P, Poole AR, Buschmann MD. Detection and analysis of cartilage degeneration by spatially resolved streaming potentials. J Orthop Res. 2002 Jul;20(4):819-26.
44. Dumont J, Ionescu M, Reiner A, Poole AR, Tran-Khanh N, Hoemann CD, McKee MD, Buschmann MD. Mature full-thickness articular cartilage explants attached to bone are physiologically stable over long-term culture in serum-free media. Connect Tissue Res. 1999;40(4):259-72.
45. http://www.biosensing.com/en/Mach1/A-class.html p.15
46. Claase MB, Grijpma DW, Mendes SC, De Bruijn JD, Feijen J. Porous PEOT/PBT scaffolds for bone tissue engineering: preparation, characterization, and *in vitro* bone marrow cell culturing. J Biomed Mater Res. 2003 Feb 1;64A(2):291-300.
47. Hollister SJ, Maddox RD, Taboas JM. Optimal design and fabrication of scaffolds to mimic tissue properties and satisfy biological constraints. Biomaterials. 2002 Oct;23(20):4095-103.
48. Temenoff, J.S., Lu, L., and Mikos, A.G. Bone-tissue engineering using synthetic biodegradable polymer scaffolds. In: Davies, J.E., ed. Bone Engineering. em sqared incorporated, Toronto, 2000, pp.454-459
49. Caplan, A.I. New logic for tissue engineering: Multifunctional and biosmart delivery vehicules. In: Davies JE, ed. Bone Engineering. em sqared incorporated, Toronto, 2000, pp. 441-445.
50. Choi BY, Park HJ, Hwang SJ, Park JB. Preparation of alginate beads for floating drug delivery system: effects of CO(2) gas-forming agents. Int J Pharm. 2002 Jun 4;239(1-2):81-91.

51. Zhao L, Chang J. Preparation and characterization of macroporous chitosan/wollastonite composite scaffolds for tissue engineering. J Mater Sci Mater Med. 2004 May;15(5):625-9.
52. Ma PX, Zhang R, Xiao G, Franceschi R. Engineering new bone tissue *in vitro* on highly porous poly(alpha-hydroxyl acids)/hydroxyapatite composite scaffolds. J Biomed Mater Res. 2001 Feb;54(2):284-93.
53. Zhang Y, Zhang M. Cell growth and function on calcium phosphate reinforced chitosan scaffolds. J Mater Sci Mater Med. 2004 Mar;15(3):255-60.
54. Harris LD, Kim BS, Mooney DJ. Open pore biodegradable matrices formed with gas foaming. J Biomed Mater Res. 1998 Dec 5;42(3):396-402.
55. Gomi K, Davies JE. Guided bone tissue elaboration by osteogenic cells *in vitro*. J Biomed Mater Res. 1993 Apr;27(4):429-31.
56. Yoshikawa T, Ohgushi H, Tamai S. Immediate bone forming capability of prefabricated osteogenic hydroxyapatite. J Biomed Mater Res. 1996 Nov;32(3):481-92.
57. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials. 1996 Jan;17(2):137-46.
58. Radder AM, Leenders H, van Blitterswijk CA. Application of porous PEO/PBT copolymers for bone replacement. J Biomed Mater Res. 1996 Mar;30(3):341-51.
59. Lahiji A, Sohrabi A, Hungerford DS, Frondoza CG. Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. J Biomed Mater Res. 2000 Sep 15;51(4):586-95.
60. Seol YJ, Lee JY, Park YJ, Lee YM, Young-Ku, Rhyu IC, Lee SJ, Han SB, Chung CP. Chitosan sponges as tissue engineering scaffolds for bone formation. Biotechnol Lett. 2004 Jul;26(13):1037-41.
61. Fakhry A, Schneider GB, Zaharias R, Senel S. Chitosan supports the initial attachment and spreading of osteoblasts preferentially over fibroblasts. Biomaterials. 2004 May;25(11):2075-9.
62. Kii I, Amizuka N, Shimomura J, Saga Y, Kudo A.Cell-cell interaction mediated by cadherin-11 directly regulates the differentiation of mesenchymal cells into the cells

of the osteo-lineage and the chondro-lineage. J Bone Miner Res. 2004 Nov;19(11):1840-9. Epub 2004 Aug 23.

63. Ferrari SL, Traianedes K, Thorne M, Lafage-Proust MH, Genever P, Cecchini MG, Behar V, Bisello A, Chorev M, Rosenblatt M, Suva LJ. A role for N-cadherin in the development of the differentiated osteoblastic phenotype. J Bone Miner Res. 2000 Feb;15(2):198-208.
64. Schiller PC, D'Ippolito G, Balkan W, Roos BA, Howard GA. Gap-junctional communication is required for the maturation process of osteoblastic cells in culture. Bone. 2001 Apr;28(4):362-9.
65. Cheng SL, Lecanda F, Davidson MK, Warlow PM, Zhang SF, Zhang L, Suzuki S, St John T, Civitelli R. Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation. J Bone Miner Res. 1998 Apr;13(4):633-44.
66. Hunter A, Archer CW, Walker PS, Blunn GW. Attachment and proliferation of osteoblasts and fibroblasts on biomaterials for orthopaedic use. Biomaterials. 1995 Mar;16(4):287-95.
67. Rothamel D, Schwarz F, Sculean A, Herten M, Scherbaum W, Becker J. Biocompatibility of various collagen membranes in cultures of human PDL fibroblasts and human osteoblast-like cells. Clin Oral Implants Res. 2004 Aug;15(4):443-9.
68. Trylovich DJ, Cobb CM, Pippin DJ, Spencer P, Killoy WJ. The effects of the Nd:YAG laser on *in vitro* fibroblast attachment to endotoxin-treated root surfaces. J Periodontol. 1992 Jul;63(7):626-32.
69. Baxter LC, Frauchiger V, Textor M, ap Gwynn I, Richards RG. Fibroblast and osteoblast adhesion and morphology on calcium phosphate surfaces. Eur Cell Mater. 2002 Sep 30;4:1-17. Print 2002 Sep 30.
70. Bowers KT, Keller JC, Randolph BA, Wick DG, Michaels CM. Optimization of surface micromorphology for enhanced osteoblast responses *in vitro*. Int J Oral Maxillofac Implants. 1992 Fall;7(3):302-10.

71. Lee JW, Kim YH, Park KD, Jee KS, Shin JW, Hahn SB. Importance of integrin beta1-mediated cell adhesion on biodegradable polymers under serum depletion in mesenchymal stem cells and chondrocytes. Biomaterials. 2004 May;25(10):1901-9.