Thesis for the Degree of Master of Science

Preparation and Characterization of Chitosan-natural Nano-Hydroxyapatite-Fucoidan Biomaterial based Nanocomposite for Bone Tissue Engineering

By

Baboucarr Lowe

Department of Marine Bio Convergence Science

Specialized Graduate School of Science & Technology Convergence

Pukyong National University

February 2016

Preparation and Characterization of Chitosan-natural Nano-Hydroxyapatite-Fucoidan Biomaterial based Nanocomposite for Bone Tissue Engineering

골 조직 공학을 위한 키토산 나노 하이드록시아파타이트-푸코이단을 기반으로 한 나노생체물질의 합성 및 특성화

Advisor: Prof. Se-Kwon Kim

By

Baboucarr Lowe

A thesis submitted in partial fulfillment of the requirements for degree of Master of Science

In Department of Bioconvergence Science

Specialized Graduate School Science & Technology Convergence

Pukyong National University

February 2016

Preparation and Characterization of Chitosan-natural Nano-Hydroxyapatite-Fucoidan Biomaterial based Nanocomposite for Bone Tissue Engineering

A dissertation

by

Baboucarr Lowe

Approved by:

Prof. Jae-Young Je (Chairman)

Prof. Hak-Jun Kim (Member)

Prof. Se-Kwon Kim (Member)

February 2016

Preparation and Characterization of Chitosan-natural Nano-Hydroxyapatite-Fucoidan Biomaterial based Nanocomposite for Bone Tissue Engineering

Baboucarr Lowe

Department of Marine Bio Convergence Science, Specialized Graduate School & Technology Convergence, Pukyong National University

Abstract

Tissue engineering processes involve the design and development of functional biomaterial substitutes which can serve as carbon copy of the extracellular matrix and produce the essential information for cells to capacitate tissue development processes. Bone tissue engineering provides us with the option to repair damage bone tissues emanating from injuries sustained as a result of bone fracture, osteoarthritis, tumor, trauma and other congenial diseases. As a result of growing demand for bone grafts, research in the area of artificial organ development using functional biomaterials as suitable prostheses in reconstructive surgery for the treatment of loss or damage tissue or organs is gaining momentum.

In this respect, Nano-Hydroxyapatite (nHA) was isolated from salmon bone via an alkaline hydrolysis. The resulting nHA was characterized using several analytical tools, including thermogravimetric analysis (TGA), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction analysis (XRD), scanning electron microscopy (SEM) and transmission electron microscopy (TEM), to determine the purity of the nHA sample. The removal of organic matter from the raw fish was confirmed by TGA. FT-IR confirmed the presence of a carbonated group and the similarities to synthetic Sigma HA. XRD revealed that the isolated nHA was amorphous in nature. Microscopic examination further revealed that the isolated nHA possessed a nanostructure with a size range of 6–37 nm. The obtained nHA interacted with mesenchymal

stem cells (MSCs) was non-toxic. Increased mineralization was observed for nHA treated MSCs compared to the control group.

Further, a solid three dimensional (3D) composite scaffold with a high potential usage for bone tissue engineering was prepared using freeze drying method composed of chitosan (C), natural nano-hydroxyapatite (nHA) isolated from salmon bones and fucoidan (F), (C-nHA-F). Fourier transform infrared spectroscopy (FT-IR), Thermal Gravimetric Analysis (TGA), X-ray Diffraction Analysis (XRD), Scanning Electron Microscopy (SEM) and Optical Microscopy (OM) were used to determine physiochemical constituents and morphology of the scaffold. The addition of nHA in the C-F composite scaffold reduce the water uptake and water retention ability. FT-IR analysis shows the presence of carbonated group in the scaffold which is due the presence of nHA that was isolated via alkaline hydrolysis from salmon fish bones. Microscopic results indicated that dispersion of nHA and fucoidan in the chitosan matrix were uniform and showed the pore size (10 to 400 μm) of the composite revealing a suitable micro architecture for cell growth and nutrient supplementation. This was further elucidated *in vitro* using Periosteum-derived Mesenchymal Stem cells (PMSCs) and revealed profound biocompatibility and excellent mineralization. Thus, we suggest that with further *in vivo* and clinical investigations, Chitosan-nHA-Fucoidan nanocomposite will be a promising biomaterial for use in bone tissue regeneration applications.

List of Abbreviations

ARS	Alizarin Red S
ATCC	American Type Culture Collection
CPC	Cetyl Pyridium Chloride
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
FBS	Fetal Bovine Serum
FESEM	Field Emission Scanning Electron Microscopy
FT-IR	Fourier Transform Infrared Spectroscopy
JCPDS	Joint Committee on Powder Diffraction Standards
MTT	(3-(4,5-dimethyl-2-yl)-2, 5-diphenyltetrazolium bromide
MSC	Mesenchymal stem cell
nHA	Nano-hydroxyapatite
ODM	Osteogenic Differentiation Medium
PLGA	Poly(lactic-co-glycolic) acid
P-MSC	Periosteum Mesenchymal Stem Cell
PLLA	Poly-L-lactide acid
rBMSC	Rat bone marrow derived mesenchymal stem cells
rhBMP-2	Recommbinant human bone morphongenic protein -2

SAED	Selective Area Diffraction Analysis
SCEM	Stem Cell Expansion Medium
TGA	Thermal gravimetric Analysis
TEM	Transmission Electron Microscope
UV	Ultra Violet Radiation
XRD	X-Ray Diffraction

Table of Contents

List of Figures

Chapter 1

1.1 . Introduction

Tissue engineering processes involve the design and development of functional biomaterial substitutes that can serve as carbon copy of the extracellular matrix and produce the essential information for cells to capacitate tissue development processes(Dvir, Timko, Kohane, & Langer, 2011). Tissue engineering-based therapeutics have employed the use and design of nanoscale technologies, which provide biochemical signaling molecules for cells and help to mimic the three dimensional (3D) extracellular matrix (ECM) in general. The ECM constitutes different molecules at nanometers level; glycoproteins, collagen and proteoglycans and glycosaminoglycan are important biomolecules that provides structural support to cells(Arora et al., 2012). Tissue engineering is a multidisciplinary field, which is a combination of basic science, engineering and medicine. A large number of different tissue engineering products being developed by researchers are still in the laboratory and clinical trial levels. Bone tissue engineering provides us with the option to repair damage bone tissues emanating from injuries sustained as a result of bone fracture, osteoarthritis, tumor, trauma (Cancedda, Dozin, Giannoni, & Quarto, 2003) and other congenial diseases(Feng, Niu, Gao, Peng, & Shuai, 2014; Gu, Huang, Rahaman, & Day, 2013). Bone is an important organ that is involved in serious remodeling processes essential to the human body(Salgado, Coutinho, & Reis, 2004). By its nature, a bone is a complex hierarchical framework largely composed of nano-hydroxyapatite (nHA), collagen and other noncollagenous materials(Bagheri-Khoulenjani, Mirzadeh, Etrati-Khosroshahi, & Ali Shokrgozar, 2013). Approximately 25% of calcified bone is composed of an organic matrix, along with 2-5% cells, 5% water, and 70% inorganic nHA. Additionally, more than 94% of the collagen is composed of osteoid (unmineralized portion of the bone matrix before bone tissue maturation)(Sommerfeldt & Rubin, 2001). Several

types of proteins (glycoproteins, proteoglycans, and sialoproteins) form part of the bone matrix **(**Sommerfeldt & Rubin, 2001) **(Figure 1).**

An estimated million cases of bone graft procedures and reconstruction happen each year. These bone defects are very difficult to manage, and many techniques have been used for this purpose(Spitzer, Perka, Lindenhayn, & Zippel, 2002). Autologous bone grafts can be used for a large number of orthopedic and reconstruction procedures resulting from tumor excision and debridement from osteomyelitis (Horch et al., 2013; Horch et al., 2012). However, these procedures have several limitations, such as the risk of transfections, increased blood loss and donor site morbidity. For allogenic bone grafts, the issue of guided bone healing becomes the most critical concern, although with the appropriate growth factors, osteogenesis for bone regeneration can be ultimately induced. However, resportion of the bone graft in both situation may reduce its mechanical strength (Younger & Chapman, 1989). As a result, suitable bone substitutes have been studied. Largely polymers, these materials have bioactive groups that can induce bone formation and are resorbable (C. Vacanti & Vacanti, 1994; C. A. Vacanti & Bonassar, 1999).

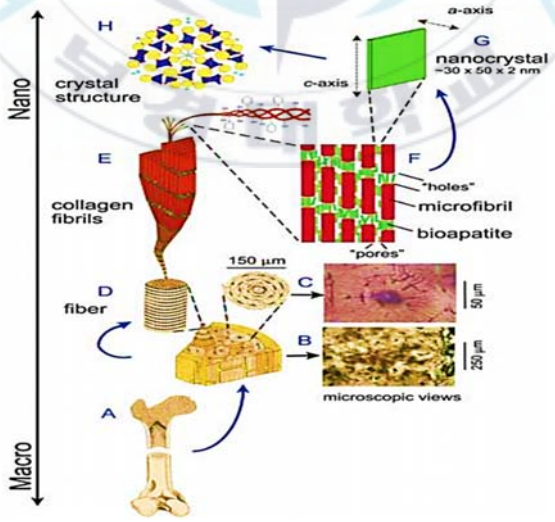

Figure 1. Hierarchical structure of bone. (A) Long bone. (B) Cross-section of cortical bone. (C) Osteon. (D) Collagen fiber. (E) The smallest unit of the organic component. (F) Microfibrils. (G) Apatite crystals. (H) Atomic structure of nHA. Figure reproduced with permission from (Allo, Costa, Dixon,

Mequanint, & Rizkalla, 2012).

Thus, in bone tissue engineering, the use of both synthetic and naturally sourced nHA is considerably attractive to tissue engineers because of its high degree of resemblance to natural bone structure(Ge et al., 2012), and nHA is largely used in the construction of mimicry 3D polymer assemblage applications and can provide the mechanical strength necessary to serve as a template structure for bone tissue regeneration, which allows cell growth and adhesion. This application is aided by the osteoconductive(B.-S. Kim, Kang, Yang, & Lee, 2014; Lin, Chen, & Chang, 2012; Malina, Biernat, & Sobczak-Kupiec, 2012), osteoinductive (Ge, Zhao, Wang, Liu, & Yang, 2013), nontoxic, nonimmunogenic and noninflammatory (Swetha et al., 2010) properties of nHA. Another encouraging technique is the use of osteogenic progenitor cells that can be isolated from the bone marrow and thoracolumbar postmortem (D'Ippolito, Schiller, Ricordi, Roos, & Howard, 1999). For example, mesenchymal stem cell (MSC) pluripotency is continually exploited for targeted stem cell differentiation and guided bone cell formation to produce tissues. Biomaterials and other polymers, when engineered with suitable scaffolds, can interact with stem cells to remodel the injured bone tissue and finally replace the affected organs. These scaffolds aim to bring cells together so that they can assemble to form tissue, allowing the effective transportation of cellular minerals for growth and development with sufficient mechanical strength(Ibara et al., 2013; Khademhosseini, Langer, Borenstein, & Vacanti, 2006; Meng et al., 2013; Michel, Penna, Kochen, Cheung, & Cheung, 2015). MSCs can be derived from variety of postnatal organs and connective tissues and have the potential to differentiate into a variety of cell lineages, such as osteoblasts (Abdallah, Haack-Sørensen, Fink, & Kassem, 2006; Bäckesjö, Li, Lindgren, & Haldosén, 2009; Chevallier et al., 2010; D.-H. Kim et al., 2015; Kulterer et al., 2007; Müller et al., 2008), chondrocytes (Bosnakovski et al., 2006; Hegewald et al., 2004; Ng et al., 2008; Worster, Nixon, Brower-Toland, & Williams, 2000), hepatocytes (Alison et al., 2000; Petersen et al., 1999), adipocytes (Kanda,

Hinata, Kang, & Watanabe, 2011; Muruganandan, Roman, & Sinal, 2009; Romanov, Darevskaya, Merzlikina, & Buravkova, 2005), endothelial cells (Romagnoli & Brandi, 2014), fibroblasts(Lee, Shah, Moioli, & Mao, 2010), marrow stroma (Tuan, Boland, & Tuli, 2003; J. Wang et al., 2015), neuron (Anghileri et al., 2008; Kopen, Prockop, & Phinney, 1999; Qu-Petersen et al., 2002) and mesangial cells(Ito, Suzuki, Okabe, Imai, & Hori, 2001). Human MSCs can be isolated and purified through many stages while retaining their properties. MSCs can be derived from adipose (Grisendi et al., 2010; W.-S. Kim, Park, Park, Kim, & Sung, 2009; Mehlhorn et al., 2007; Zannettino et al., 2008; Zuk et al., 2001), synovial membrane (De Bari, Dell'Accio, Tylzanowski, & Luyten, 2001; De Bari et al., 2003; Djouad et al., 2005; Jones et al., 2008), bone marrow (Bühring et al., 2009; Battula et al., 2007; Bernardo et al., 2007; Horwitz et al., 2002; Pittenger et al., 1999; Studeny et al., 2002), periosteum (Caballero, Reed, Madan, & van Aalst, 2010; De Bari, Dell'Accio, & Luyten, 2001; Eyckmans & Luyten, 2006; Ferretti et al., 2012; Hui, Li, Teo, Ouyang, & Lee, 2005; Nakahara, Goldberg, & Caplan, 1991; Ringe et al., 2008; Q. Wang, Huang, Zeng, Xue, & Zhang, 2010; Yoshimura et al., 2007; Zheng et al., 2006), dermis (Bartsch Jr et al., 2005; Hoogduijn et al., 2007; Hsiao et al., 2011; Joannides et al., 2004; Riekstina et al., 2009; Riekstina, Muceniece, Cakstina, Muiznieks, & Ancans, 2008; Vaculik et al., 2012; Young et al., 2001; Z. Zhao, Liao, Cao, Jiang, & Zhao, 2005), pericytes (Brighton et al., 1992; Diefenderfer & Brighton, 2000; Reilly, Seldes, Luchetti, & Brighton, 1998), blood (Flynn, Barry, & O'brien, 2007; Gang et al., 2006; Kang et al., 2005; S. M. Kim et al., 2008; M. Wang et al., 2009; Zvaifler et al., 2000) and trabecular bone tissue (Gimble & Guilak, 2003; Nöth et al., 2002; Osyczka, Nöth, Danielson, & Tuan, 2002; Sakaguchi et al., 2004; Song, Young, Webb, & Tuan, 2005; Sottile, Halleux, Bassilana, Keller, & Seuwen, 2002). The ability for these stem cell lines to produce tissues, such as bone, fats, cartilage other connective tissues, makes them an important regenerative element for diseased or injured tissues(Horwitz et al., 1999; Pittenger et al., 1999; Sekiya, Larson, Vuoristo,

Cui, & Prockop, 2004; Van den Bos et al., 1997). MSCs suffer no immunogenic rejection when introduced, allowing large-scale expansion, characterization and the ability to ascertain growth levels, which makes them a strong candidate for use in cellular therapeutics (H. Wang et al., 2007). Scaffolds with an ideal microarchitecture stability (porosity, biocompatibility) enhance multiple biological and biophysiological requirements that can efficiently optimize bone regeneration (Li Jin, Wan, Shimer, Shen, & Li, 2012; Nukavarapu, Kumbar, Merrell, & Laurencin, 2014).

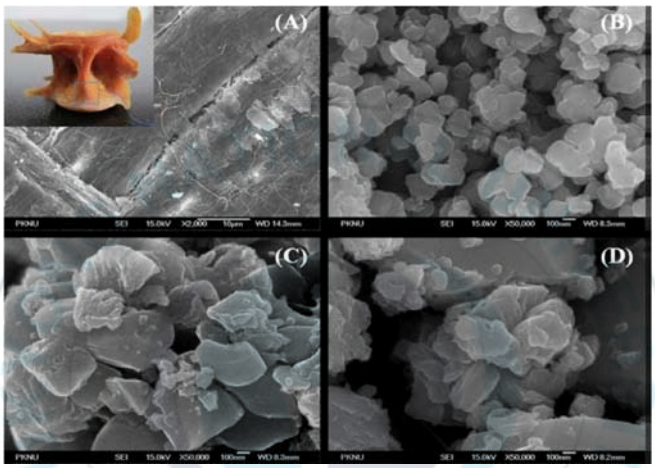

Figure 2. nHA isolated from Tuna Obessus via thermal calcination. Reproduced with permission from (J. Venkatesan & S. K. Kim, 2010).

1.2 . Nano-hydroxyapatite from natural source

Hydroxyapatite (HA) can be readily isolated from the natural bone structures of animals, such as that in fish waste (Boutinguiza et al., 2012; M Ozawa, Satake, & Suzuki, 2003; Masakuni Ozawa & Suzuki, 2002; J. Venkatesan & S. K. Kim, 2010), **(Figure 2),** pig bone (GĂśtz et al., 2011; Haberko et al., 2006; Janus, Faryna, Haberko, Rakowska, & Panz, 2008), eggshells (Sanosh, Chu, Balakrishnan, Kim, & Cho, 2009), chicken bone (Rajesh, Hariharasubramanian, & Ravichandran, 2012) and other animal hard tissues (Lü, Fan, Gu, & Cui, 2007). Naturally derived HA has a carbonated group, and several authors have reported that it possesses higher osteoconduction, bioresorption and biocompatibility properties, compared with synthetic nHA (Jayachandran

Venkatesan, Qian, Ryu, Kumar, & Kim, 2011). (Landi, Celotti, Logroscino, & Tampieri, 2003) Osteointegration between bone tissues has been elucidated to be efficient (Orr, Villars, Mitchell, Hsu, & Spector, 2001). Despite the remarkable ability of this ceramic phosphate polymer to mimic bone ECM for tissue engineering purposes, nHA has less compressive strength (brittle and low fracture toughness) for load-bearing bone repairs(Eshraghi & Das, 2012). However, using appropriate technologies, it can be readily available bioactive construct for bone tissue engineering applications (H. Liu et al., 2015). **(Figure 3)**

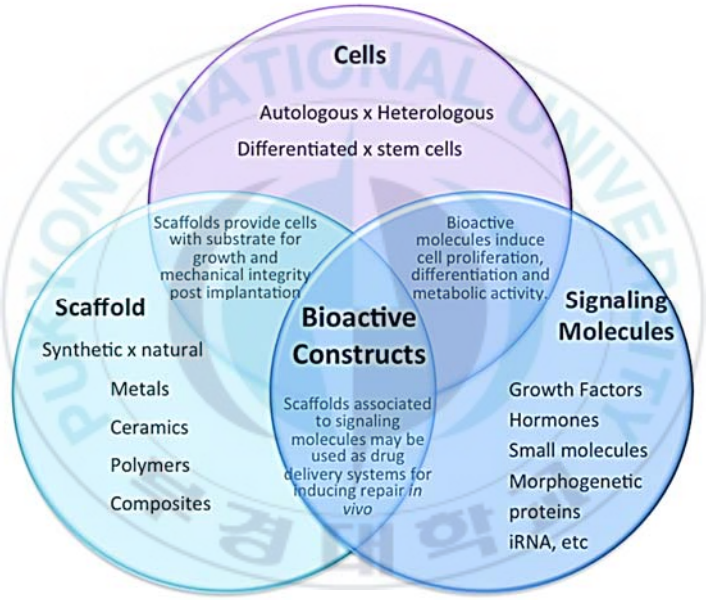

Figure 3. Scheme of Tissue engineering. Reproduced with permission from (Carvalho, de Goes, Gomes, & de Carvalho, 2013).

The desire for biodegradable polymers has been a standing one for bone tissue engineering, as they can be resorbed by the body and support tissue growth, as well as remodel damaged functions in tissues within a shorter time period. (Planell, Best, Lacroix, & Merolli, 2009) (Hickey, Ercan, Sun, & Webster, 2015; Mohanty, Misra, & Hinrichsen, 2000; Suntornnond, An, Yeong, & Chua, 2015; Tsai, Chen, Chien, Kuo, & Wang, 2014; Watson, Kasper, Engel, & Mikos, 2014; Wu, Liu, Yeung, Liu, & Yang, 2014) Additionally, biodegradable polymers are

advantageous in easing immunogenic complications related to introducing foreign biomaterials in host tissues, (Salgado et al., 2004) and their bioactive groups enhance strong bond integration with bone. Other biodegradable synthetic polymers offer efficient lot-to-lot consistency and may be easily shaped to achieve specific dimensional structures (Y. Liu, Lim, & Teoh, 2013).

1.3. Preparation of nHA by synthetic methods

nHA has been prepared by various methods to suit its usage as implant for biomedical or industrial applications. Using a calcium nitrate tetrahydrate along with ammonium dibase phosphate in the presence of polyacrylic acid, (S Zhang & Gonsalves, 1997) synthesized rod-shape nHA, and the products were hydrothermally treated at 130°C and subsequently dried. A sol-gel precipitation method using calcium nitrate tetrahydrate and potassium dihydrogen phosphate as chemical precursors has been reported with ammonia used as a pH stabilizer; the recovered nHA calcined at 600°C (Sanosh, Chu, Balakrishnan, Lee, et al., 2009). nHA can also be produced via a chemical process involving the use of Schiff bases as complexing agents. In this method, the Schiff bases are first synthesized by reacting carbonyl compounds (acetylacetone and 2-hydroxybenzophenone) and diamines (2-aminophenol, 2,2-dimethyl-propylendiamine and 1,8-diamino-3,6-dioxaoctane) in methanol solution and continual refluxing at thermal conditions of 70°C, followed by the crystallization and filtration of the reaction mixture. To synthesize the nHA crystals, calcium nitrate tetrahydrate is added in to the Schiff base solution containing methanol. This step is followed by the addition of ammonium phosphate and sodium hydroxide to increase the pH. Finally the reaction mixture is heated to 120°C, cooled, centrifuged and washed to obtain the crystals (Mohandes, Salavati-Niasari, Fereshteh, & Fathi, 2014). Using a wet chemical method reacting 1 mol of calcium hydroxide and 0.6 mol orthophosphoric acid, nHA has been synthesized using a radio frequency plasma process. The precipitation reaction is stabilized with H_3PO_4 and further

dried to remove moisture. This method can produce nHA in the size range 10-100 nm (J. Xu et al., 2004). **(Figure 4)**

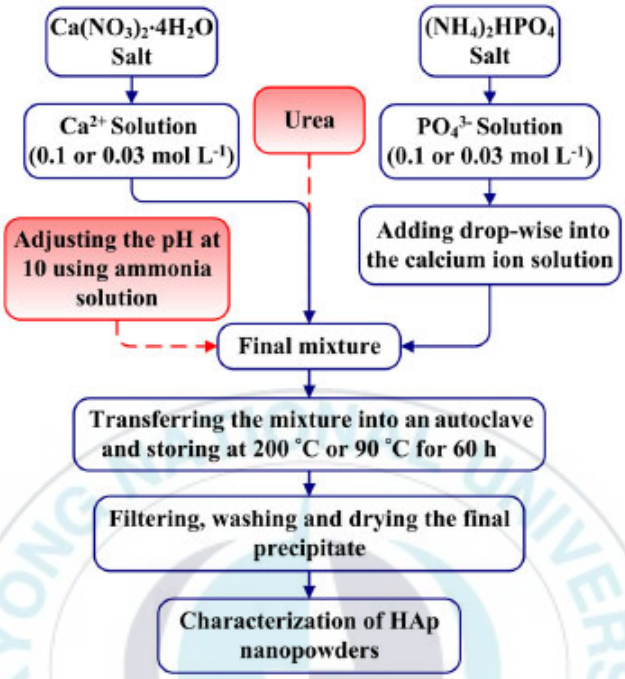

Figure 4. Synthesis of nHA by a hydrothermal process. Figure reproduced with permission from (Sadat-Shojai, Atai, & Nodehi, 2011).

Furthermore, (G. Guo, Sun, Wang, & Guo, 2005) reported the synthesis of nHA via reverse microemulsion (using TX-100 and Tween 80) with *n*-butanol and *n*-hexanol as mixed and mixed cosurfactants, respectively, which was compared with a conventional precipitation using 0.3 M $(NH_4)_2HPO_4$ and 0.5 M $Ca(NO_3)_2$. nHA produced by the microemulsion method formed a smaller particle size and agglomeration, compared with the precipitation method (G. Guo et al., 2005). In the ultrasonic precipitation method, a carbamide is use as precipitator with calcium nitrate and ammonium phosphate as starting materials. A sonic horn is dipped into the reaction mixture, and sonic power is generated. A12 wt.% NH_2CONH_2 solution is added to maintain the pH at a near neutral value, and the precipitate is filtered, washed and dried under vacuum, (Cao, Zhang, &

Huang, 2005) thus producing fine crystals of n HA particles. Microwave assisted-irradiation techniques (Mishra, Srivastava, Asthana, & Kumar, 2012), the use of cationic surfactants (J. Coelho, Moreira, Almeida, & Monteiro, 2010), hydrothermal method (H.-b. Zhang, Zhou, Li, & Huang, 2009), polymer-enhanced methods(Tseng, Kuo, Li, & Huang, 2009) and other methods(Aleksandar et al., 2013) are employed in the synthesis of nHA. To model the nanosized particles efficiently, capping agent assistance (Pramanik, Tarafdar, & Pramanik, 2007) and organic modifiers (A. Wang et al., 2007) are introduced **(Figure 5).**

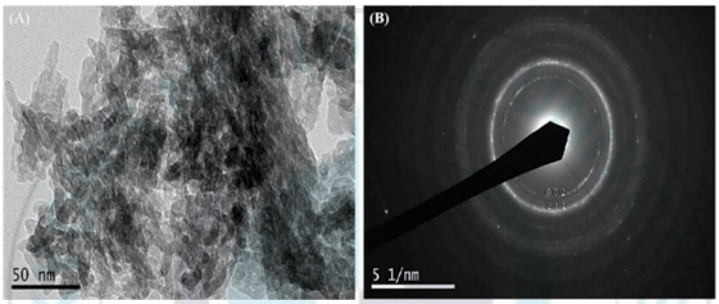

Figure 5. Transmission electron microscopy images of nHA. (A) nHA from salmon fish bone and (B) selective area diffraction of nHA. Reproduced with permission (J. Venkatesan et al., 2015).

1.4. Stem cells

Stem cells are undifferentiated biological cells that can be differentiated into specific cells. They are currently used to treat several diseases, including diabetes, arthritis, cancer, bone and tooth conditions, wound healing, etc. (Figure 6). In recent years, significant developments have been achieved through the combination of stem cells with nanomaterials for several biomedical applications (S. Hu et al., 2015; Linhua Jin et al., 2015; J. Wang et al., 2015; Zhong, Li, & Li, 2015). Bone tissue engineering is an important research area using materials with stem cells to create artificial bone for use in defective areas. Different types of stem cells, including embryonic stem cells, bone marrow-derived mesenchymal stem cells, adipose-derived stem cells, blood-derived

mesenchymal stem cells, muscle-derived stem cells and dental pulp stem cells have been used extensively. These cells have the capacity to differentiate into osteogenic lineages (Bianco & Robey, 2001; Gao et al., 2015; Grzesiak, Kolankowski, & Marycz, 2015; Huri, 2015; F. Liu et al., 2015; Mauney, Volloch, & Kaplan, 2005; Meinel et al., 2004; Seong et al., 2010; F. Wang, Zhang, Wang, & Liang, 2015; T. Zhang et al., 2015).

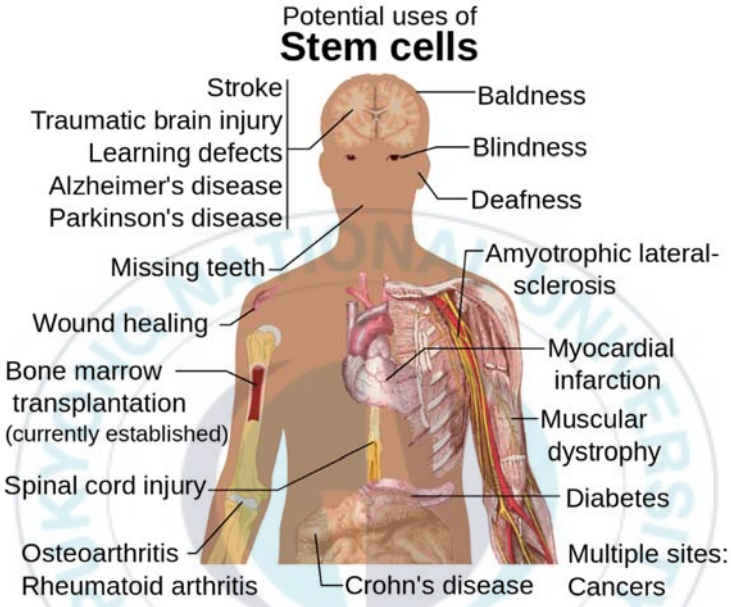

Figure 6. Potential uses of stem cells. Diseases and conditions for which stem cells treatment is being investigated (Häggström, 2014).

1.5. Stem cell interactions with nHA

The use of stem cells for bone regeneration and tissue engineering-based therapeutics is currently an important facet of regenerative medicine. Over the years, quite a significant number of studies have been spearheaded using stem cells for the regeneration of tissues and the development of organs. nHA can be functionalized with several polymers to increase its suitability for bone tissue engineering applications. Chitosan (Frohbergh et al., 2012; Koç, Finkenzeller, Elçin, Stark, & Elçin, 2014; Sellgren & Ma, 2012; Thein-Han & Misra, 2009; F. Wang, Zhang, Zhou, Guo, & Su, 2014), poly(lactide-co-glycolide)(Amna, Hassan, Khil, & Hwang, 2014; Hile, Amirpour, Akgerman, & Pishko, 2000; Y.

Hu, Hollinger, & Marra, 2001; Lao, Wang, Zhu, Zhang, & Gao, 2011; Meese, Hu, Nowak, & Marra, 2002; Shea, Smiley, Bonadio, & Mooney, 1999; Sheridan, Shea, Peters, & Mooney, 2000; Whang, Goldstick, & Healy, 2000; P. Zhou et al., 2014; Ziegler, Mayr-Wohlfart, Kessler, Breitig, & Günther, 2002), nanosilk (X. Huang et al., 2014), dextran (Schlaubitz et al., 2014), gelatin(Nair et al., 2015; Peter et al., 2010; Vozzi et al., 2014), poly(ε-caprolactone) (PCL) (Di Liddo et al., 2014)·(Ambre, Katti, & Katti, 2015; Ko, Myung, & Kim, 2015; Phipps, Clem, Grunda, Clines, & Bellis, 2012), poly(l-lactide-co-ε-caprolactone)(Akkouch, Zhang, & Rouabhia, 2014), poly-3-hydroxybutyrate-co-3-hydroxyvalerate(Sai Zhang, Prabhakaran, Qin, & Ramakrishna, 2015b), carrageenan (Hongyan Liu et al., 2014), alginate (Awad, Wickham, Leddy, Gimble, & Guilak, 2004; Hwang et al., 2009; Tang, Chen, Weir, Thein-Han, & Xu, 2012; L. Zhao, Weir, & Xu, 2010), collagen (Behravesh & Mikos, 2003; H. J. Kim, Kim, Vunjak-Novakovic, Min, & Kaplan, 2005; Liao, Cui, Zhang, & Feng, 2004; Padmanabhan et al., 2015; Tan, Krishnaraj, & Desai, 2001) and polyamide (H. Wang et al., 2007) have been reported for bone tissue engineering. These functionalized polymers can be used in different grades as in injectable forms, and others are load-bearing materials, depending on the site of application. Most of these polymers are effectual for use in bone regeneration applications either alone or with supplemented growth factors such as bone morphogenetic proteins (BMPs)(Bianco & Robey, 2001). The craniofacial complex contains sensitive tissues, and their effective regeneration has been a complex issue to many clinical experts, largely depending on the structural integrity, defect site and shape. To successfully regenerate or repair these bone structures, specially designed injectable grafts have proven to be highly effective. Chitin-PCL-nHA microgels that have elastic modulus with thermal stability can be injected into defective sites together adipogenic MSC protein adsorption to elicit osteogenic differentiation (Arun et al., 2015) suitable for craniofacial repair/regeneration. Additionally, Frohbergh *et al*. reported that crosslinked nanofibers of chitosan-electrospun nHA with genipin might be

suitable for cranial and maxillofacial reconstruction (Frohbergh et al., 2012). This scaffold exhibited a Young's modulus of 147.4±21.7 MPa at a 2.0% nHA concentration. nHA silica gel-enriched platelet growth factors with MSCs appears to be an effective amalgamation for bone regeneration. (Behnia et al., 2013) Histomorphometric analysis of one group demonstrated 29.45% and 44.55% ($p < .05$) increases in 6 and 12 weeks, respectively, which were higher than results from other groups at standard experiment times **(Figure 7).**

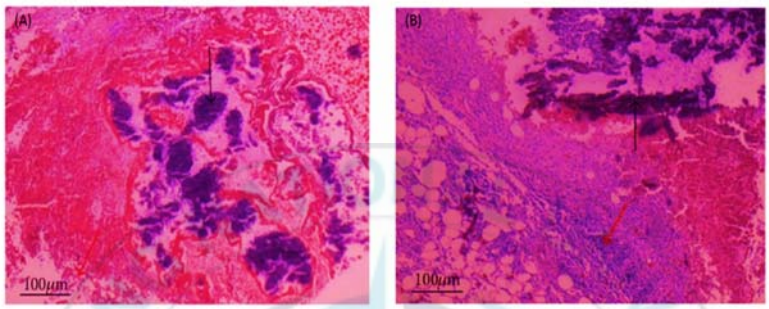

Figure 7. Light microscopy images of chitosan/nHA/rBMSC-induced bone formation at 2 and 4 weeks after implantation, 100X. (a) HE, 2 weeks; (b) HE, 4 weeks. (Yu et al., 2013)

1.6. nHA as delivery vehicles

Nanostructured surfaces have been used as delivery carriers for drugs to tumor (Motskin et al., 2009) cells and growth factors (H. H. Xu, Weir, & Simon, 2008) for bone regeneration. The excellent candidacy of these nanostructured systems is due to their unique physical properties of mechanical, optical, and electrical significance and serve as unique delivery platforms for biochemical applications in various ways to perform targeted biochemical functions (Emerich & Thanos, 2003). The integration of proteins/growth factors, such as transforming growth factor beta (TGF-ß), bone morphogenetic proteins (BMP), insulin-like growth factor (IGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) into scaffolds can control osteogenesis and the differentiation of osteoprogenitors cells into their respective lineages. In this process, bone tissue

formation is enhanced through the modelling of the extracellular matrix **(Figure 8)**.

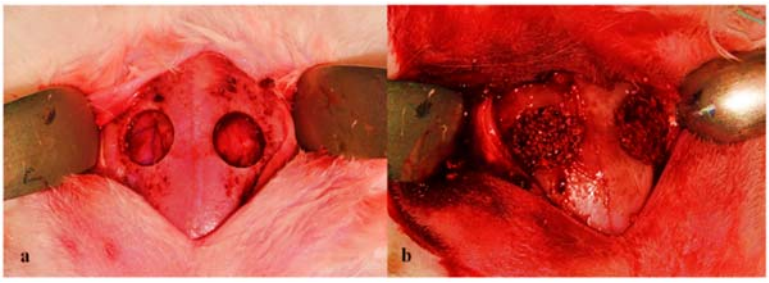

Figure 8. (a) Calvarial defect sites and (b) defect sites implanted with or without Bio-Oss® or BMP-2/Bio-Oss®. Figure reproduced with permission from (Huh et al., 2015).

(Bose, Roy, & Bandyopadhyay, 2012). Additionally, in bone tissue engineering, this enhancement can be largely attributed to the fact that nanoscaffolds exhibit controlled absorbency and high pore definition. With proper MSC adherence, these features promote more differentiation in autogenous bone grafts, compared with MSC delivery on synthetic or natural scaffolds (Drosse et al., 2008; El Tamer & Reis, 2009; Phinney & Prockop, 2007; Slater, Kwan, Gupta, Panetta, & Longaker, 2008; Torroni, 2009; Woo et al., 2007). Similarly, with the addition of recombinant human BMP 2 (rhBMP-2) and biological carrier materials such as fibrin, cell culture, autologous serum, fibrovascular tissue growth and osteoclastic activity was revealed to be higher in autologous serum in a sheep model (Boos et al., 2014). Electronspun poly(lactic-c-glycolide)/amorphous nanocalcium phosphate was able to efficiently optimize the physical and biological properties of a scaffold for bone repair/reconstruction due to its biocompatibility and biodegradability (Hild et al., 2011; Loher et al., 2006; Scherberich, Müller, Schäfer, Banfi, & Martin, 2010; Schneider et al., 2008). With human adipose-derived stem cells, the material elicited homogenous vascularization by avian vessels of chick chorioallantoic membrane (CAM) within 7 days (Buschmann et al., 2012) and is suitable for use as a bone reconstruction scaffold.

Osteoclasin expression is believed to be stimulated by the number of low nanoparticles per unit volume, which makes it a potential candidate that can be used to functionalize other bone tissue engineering polymers in the form of a composite. Low-level vascularization could result in a necrotic core, which is an important challenge/limitation in bone reconstruction tissue engineering-based therapeutics (Laschke et al., 2006; Scherberich et al., 2010). Further, hydrolytically engineered/produced copolymers of PLGA (poly(lactic-co-glycolic acid)) for bone tissue engineering have been widely used in the reconstruction/regeneration of bone tissue and is often cross-linked with calcium phosphate-containing polymers, etc., although associated occurrences of tissue inflammation have been reported (N.-J. Chang et al., 2012). The functional roles of these polymers include supporting the adhesion and differentiation capacities of stem cells, which become more activated after interacting with osteoinductive medium containing cytokines, as matrix-bound cytokines have stromal effects on hematopoietic stem cell lines. (Calvi et al., 2003) Beta-glycerophosphate, dexamethasone and ascorbate have been used to differentiate stem cells to osteogenic lineages (Pittenger et al., 1999). This has also been elucidated *in vivo* through early calcification and osteoid tissue formation when used as a composite in bone orthopedic implants (Fricain et al., 2013). Procine acellular dermal matrix is an important polymeric collagen-based functional biomaterial with stromal and osteogenic differentiation capacity. Its high enzymatic degradation rates, together with a strong compressive modulus, can be achieved when assembled with nHA **(Figure 9).**

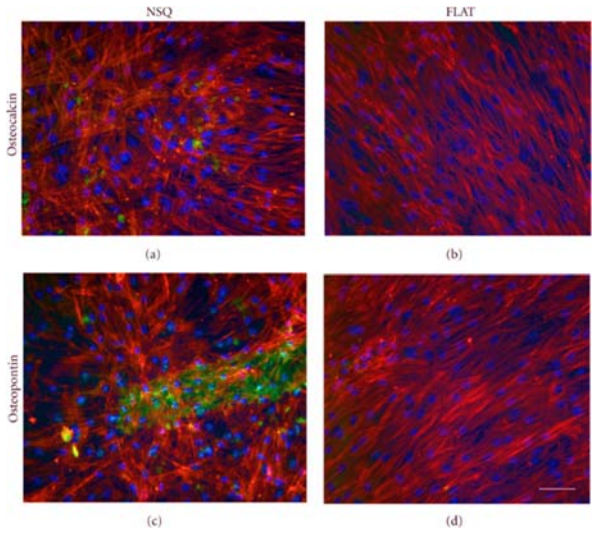

Figure 9. Bone protein expression in skeletal stem cells cultured on osteoinductive nanotopography. Figure reproduced with permission from (McNamara et al., 2010).

nHA stabilizes the collagen-matrix surface by providing inherent compressive strength of mechanical durability that is suitable for periodontal tissue engineering (Ge et al., 2013). This assemblage process is possible via a biomimetic chemical method reported by (J. Guo et al., 2013). This biomimetic mineralization approach of using nanoapatite layers is efficient in manipulating the structural surface chemistry and geometry of conjugated polymers and could enhance stem cell differentiation to an osteoblast lineage for tissue engineering applications(Ge et al., 2012). Researchers(Beom-Su Kim et al., 2013) have reported a coprecipitation synthesis method for generating chitosan/nHA fabric via a wet spinning method. Much greater *in vitro* proliferation was observed for chitosan/nHA fabric with human MSC (hMSCs), which might be due to the fiber diameter; initial cell adhesion is correlated to the surface area of the fiber (Chen, Patra, Warner, & Bhowmick, 2007). The bioactive group possessed by nHA and the excellent mechanical strength of polyamide forms a biocompatible combination that can be used for bone regeneration by promoting osteogenesis and that has generated promising results both *in vitro* and *in vivo* (J. Li et al., 2010). Kuznetsov *et al*., demonstrated *in vivo* bone formation from marrow

stromal fibroblasts using immunodeficient mice and hydroxyapatite-tricalcium phosphate ceramic as a delivery vehicle. After 8 weeks, 58.8% single colony-forming strains formed bone (Kuznetsov et al., 1997). Meanwhile, an *in situ* chitosan/nHA/collagen gel elicited good histocytological compatibility in the delivery of rat bone marrow mesenchymal stem cells (rBMSCs), with enhanced immunosuppressive properties, cell growth within the matrices of the gel and slight inflammation, compared with the control without rBMSCs. (Z. Huang et al., 2011) This delivery system can be targeted for further differentiation of MSCs for bone tissue formation by adding growth factor and differential medium **(Figure 10).**

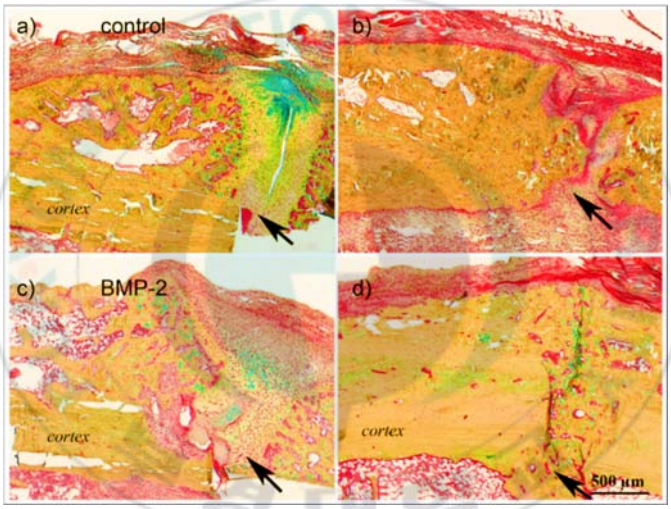

Figure 10. Histological staining of the calluses of the control and the BMP-2 group at days 28 and 84. Movat pentachrome stainings of the two groups (control group: a, b; and BMP-2 group: c, d) at days 28 (a, c) and 84 (b, d).The arrows point to the osteotomy gap. At day 28, the calluses of both groups showed no fully mineralized bridging, as fibrous tissue was still filling the gap above the osteotomy. After 84 days, the healing progressed, with a fully mineralized callus in the BMP-2 group. Scale bar: 500 µm. Figure reproduced with permission from (Faßbender, Minkwitz, Strobel, Schmidmaier, & Wildemann, 2014).

Additionally, a nHA-coated electrospun poly-L-lactic acid (PLLA) scaffold induced osteogenesis in umbilical cord blood-derived unrestricted somatic stem cells by inducing osteogenic differentiation, as elucidated by the expression of osteogenic markers (Seyedjafari, Soleimani, Ghaemi, & Shabani, 2010).

Future directions involve the development of artificial constructs using nHA in regenerative medicine. A huge economic burden is associated with the treatment of patients with bone fractures, and with a growing aging population (in South Korea from 3.1% in 1997 to 13.1% in 2015),(M. Y. Kim, Im, & Park, 2015) metabolic bone diseases, such as osteoporosis, are expected to rise globally. Research and development in the fields of biomedical technologies and biomaterials are providing alternative choices for orthopedic surgeons with the possibility to repair and or regenerate organs that have suffered from injury, disease or as a result of aging(Jackson & Simon, 1999). Traditional methods of bone regeneration through orthopedic procedures require time spent performing surgery to harvest autografts, and vascularized grafts would require immense microsurgical operative techniques with sophisticated infrastructures (Burg, Porter, & Kellam, 2000). In light of these challenges, novel strategies using biomaterials and other synthetic polymers for tissue regeneration through bioinspired techniques is reshaping both laboratory and clinical research efforts for developing bone tissues.

Nanotechnology-based approaches in regenerative medicine involve the isolation of nanobiomaterials, characterization, functionalization, *in vitro* and *in vivo* investigations as well as clinical trials. Nanotechnology researcher have dug deeply into an interdisciplinary spectrum of combined studies in the fields of medicine, material science, biotechnology, biology, chemistry and molecular biology. Nanotechnology-based engineering of biological systems is expected to contribute to a global surge in the quality of human health. Nanotechnology holds much promise in providing solution to these health problems. To ensure efficiency and meet therapeutic demands in clinics and meet the demands of reconstructive surgery procedures, there is a need to develop advanced

mechanisms to elicit greater biochemical interaction capabilities with material systems. Using nanotechnological strategies in scaffold fabrication a higher level can be attained. Stem cell nanoengineering and cell-based therapy are essential components of the overall process and need to be observed closely. To recapitulate, advances in the engineering and design of micro/nanotechnologies for scaffold fabrication would provide a throughput in the discovery and patterning of stem cells to elicit alignments for enhanced tissue formation. Additionally, nHA has weak mechanical strength, which limits its direct usage as a scaffold for the regeneration of load-bearing tissues **(Figure 11).**

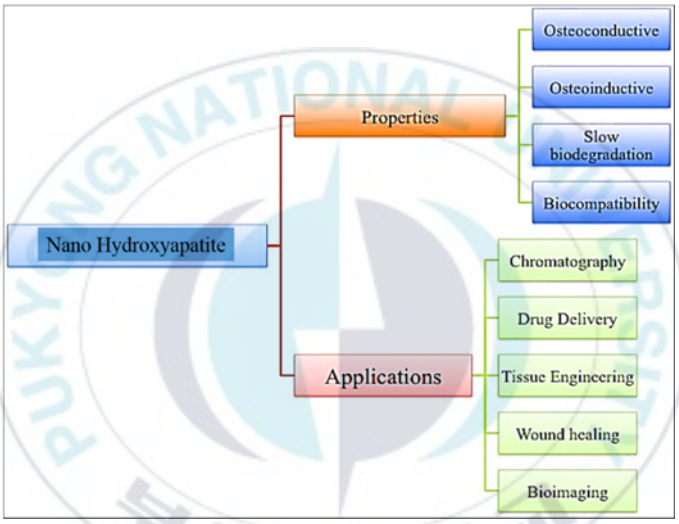

Figure 11. Properties and applications of nHA.

1.7. Future directions

We suggest that individual subunits of polymeric surfaces and their interactions with biological molecules, such as proteins and growth factors, be explored; this research area can yield primary information to increase the rate of targeted differentiation of stem cells. This knowledge would pave the way for greater advances in stem cell-based therapeutics. Additionally, engineering and introduction of nanocarriers would ease the challenges of reaching target sites that are not easily accessible, such as blood-brain barrier, tight junctions and capillaries (Arora et al., 2012). For implants to more efficiently serve the purpose of tissue regeneration and reconstruction mechanisms for injuries and

cancers, surface coating of polymeric material with stronger metallic elements can substantially increase strength, and cell-material surface reactions can create a dynamic environment for tissue regeneration. Further, we anticipate a future in medical therapeutics in which nanotechnologies will catalyze responses to the most critical tissue regeneration processes by producing inspired nanosystems that can enhance tissue formation. We hope nHA will become an important player toward this reality. Matching the scaffold materials' target properties for stem cell differentiation and tissue regeneration would change the course of regenerative procedures in tissue engineering. The use of stem cells in tissue regeneration will likely continue to account for a large number of procedures in tissue damage repair in the future. In our continued quest to understand the complex biological nature of stem cells and their dynamics when they interact with polymers, we hope to develop strategic protocols/mechanisms to ensure better cell-to-cell coordination in tissue formation processes, such as aligning stem cells in a specific course within the framework of the microenvironment of scaffolds and/or composites. Additionally, with the inclusion of targeted differentiation-producing minerals, cellular integration and proliferation may become much more pronounced with nHA.

1.8. Conclusion

Bone tissue engineering is an indispensable way to efficiently address therapeutic needs for a large number of orthopedic surgeries. This technology is eminent because of the huge potentials of biomaterials in restoring tissue and organ functions and/or repairing damage. With biomaterials, tissue configuration can be restored, providing a lasting form of support for healing and full-fledged load-bearing capacities of bone. nHA can be isolated and easily functionalized with other polymers for the fabrication of highly porous 3D scaffolds with sufficient mechanical strength, and it can furthermore serve as an osteoinductive element for targeted differentiation of MSCs for bone cell formation. nHA can serve as a delivery vehicle for bioactivators *in vitro* and *in vivo* in cellular-based therapeutics, especially for targeted stem cell

differentiation. The osteogenic potentials of MSCs are an essential element for restoring diseased bone tissues, and their easy expansion and nonimmunogenicity are reliable features for this application. In the future, keywords in the field of tissue engineering will continue to accommodate and integrate more research concepts from the fields of nanotechnology, stem cell biology and gene therapy to meet the needs and clinical demands of the 21st century.

Chapter 2.

Isolation and characterization of nanohydroxyapatite from salmon fish bone[1]

In this work, Nano Hydroxyapatite (nHA) was isolated from salmon bone using alkaline hydrolysis method. The obtained nHA was characterized using different analytical tools such as thermo gravimetric analysis (TGA), Fourier transform infrared spectroscopy (FT-IR), X-Ray Diffraction analysis (XRD), Scanning Electron Microscopy (SEM) and transmission electron microscopy (TEM) to determine the purity of the nHA. Removal of organic matter from

[1] This work has been published as Venkatesan, Jayachandran, Baboucarr Lowe, Panchanathan Manivasagan, Kyong-Hwa Kang, Elna P. Chalisserry, Sukumaran Anil, Dong Gyu Kim, and Se-Kwon Kim. "Isolation and Characterization of Nano-Hydroxyapatite from Salmon Fish Bone." *Materials* 8, no. 8 (2015): 5426-5439.

the raw fish bone was confirmed by TGA results. FT-IR results confirmed the presence of carbonated group and closely mimic with synthetic sigma HA. XRD XRD results revealed the isolated nHA were in amorphous in nature. Microscopy results of the isolated nHA are in nanostructure with range 6-37 nm. nm. In addition, the obtained nHA were interacted with mesenchymal stem cells cells (MSCs) and found be non-toxic. Further, increased mineralization was observed with nHA treated MSCs compared to the control group. From these results, nHA derived from salmon will be promising biomaterials in the field of bone tissue engineering.

2.1. Introduction

The increased need for organ replacements and repairs is a major challenge in human health worldwide. The treatment of damaged tissue is typically performed using autologous and allogenic grafts. These methods are limited by insufficient donors and the high risks of disease transmission. (Gomes, Leonor, Mano, Reis, & Kaplan, 2012; Salgado et al., 2004). Significant achievements in the field of tissue engineering include artificial prostheses that can treat loss, failure or regenerate tissues and/ or organs (Jayachandran Venkatesan, Ryu, Sudha, & Kim, 2012). These advances would not have been realized without the contributions of biomaterials in the form of scaffolds that meet the requirements needed for optimal tissue formation (Rahaman et al., 2011). Currently, natural biomaterials are playing pivotal roles in the design and production of biocompatible prostheses, biomimetics, elucidating specific cell functions, allowing cell-cell interactions and subsequently forming an organize matrices for tissue regeneration (Hubbell, 1995).

Biomaterials resembling the properties of bone has been continuously studied for use in bone tissue engineering (Gelinsky, Welzel, Simon, Bernhardt, & König, 2008; Matsuura et al., 2009; Pek, Gao, Arshad, Leck, & Ying, 2008; Xia et al., 2013; Yunoki et al., 2011). The use of composites consisting calcium phosphates and type I collagen is one favorable approach to mimic the extracellular matrix of bone tissue (Hoyer et al., 2012). Hydroxyapatite

ceramics [HA, $Ca_{10}(PO_4)_6(OH)_2$] are biocompatible, and their bioactivity can strengthen bone-bond formation with other tissues in an osteoconductive mechanism (X. Wang, Li, Wei, & De Groot, 2002). Nano hydroxyapatite (nHA) can be produced in different ways including synthetic methods and from natural sources. Synthetic methods used to fabricate nHA include precipitation (Cao et al., 2005; J. Zhou, Zhang, Chen, Zeng, & De Groot, 1993), radio frequency thermal plasma (J. Xu et al., 2004), reverse micro emulsion (G. Guo et al., 2005), emulsion liquid membrane system (Jarudilokkul, Tanthapanichakoon, & Boonamnuayvittaya, 2007), sol gel method (Cao et al., 2005) and hydrothermal methods (H.-b. Zhang et al., 2009). However, the synthetic production of nHA is often requires the use of hazardous chemical, ageing processes and imbalance stiochemitric ratio. HA can be isolated from bovine (Bahrololoom, Javidi, Javadpour, & Ma, 2009; Barakat, Khil, Omran, Sheikh, & Kim, 2009; Janus et al., 2008; Joschek, Nies, Krotz, & Göpferich, 2000; Ooi, Hamdi, & Ramesh, 2007; Ruksudjarit, Pengpat, Rujijanagul, & Tunkasiri, 2008; Sofronia, Baies, Anghel, Marinescu, & Tanasescu, 2014), fish scales (Kongsri, Janpradit, Buapa, Techawongstien, & Chanthai, 2013; Panda, Pramanik, & Sukla, 2014; Zainon et al., 2012), fish bone (T. Coelho et al., 2007; Lima, Weinand, dos Santos, Paesano, & Ortega, 2005; M Ozawa, Hattori, & Satake, 2007; M Ozawa & Kanahara, 2005; M Ozawa et al., 2003; Masakuni Ozawa & Suzuki, 2002; Prabakaran & Rajeswari, 2006; J. Venkatesan & S. K. Kim, 2010; Jayachandran Venkatesan, Qian, Ryu, Thomas, & Kim, 2011), *Lates calcarifer* (Kongsri et al., 2013), cuttle fish bone (B.-S. Kim et al., 2014), cat fish bone (Chattanathan, Clement, Kanel, Barnett, & Chatakondi, 2013; B.-S. Kim et al., 2014), cod fish bone (Piccirillo, Silva, Pullar, Braga da Cruz, et al., 2013). Bovine and pork origins are often associated with disease transmission and religious sentiments (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). Fish sources which are presumably much safer and the wide evolutionary gap between fish and humans suggests a low risk of disease transmission (Hoyer et al., 2012). Additionally, fish by products are

abundant, and an application of these byproducts suitable for biomedical application will reduce environmental pollution and the threats of biohazards to humans. Mesenchymal stem cells (MSCs) have capacity to differentiate into osteoblast, chondrocytes, adipocytes, muscle cells or nerve cells *in vitro* and *in vivo*. They are also studied as a common cell source for bone tissue engineering applications (Nishida et al., 2004; Shimizu et al., 2007).

We developed nHA directly from salmon fish bone through alkaline hydrolysis method to mimic the extracellular matrix of bone. Thus, we have isolated nHA from salmon fish bone. Carbonated nHA was produced using the alkaline hydrolysis method. Characterization of the isolated nHA revealed that it is amphorous with nanoparticle sizes that range from 6-37nm. We investigated the interaction between MSCs and nHA from salmon bone. The results demonstrated an increased biomineralization, possibly induced by the nHA, thus, elucidating the differentiation capacities MSCs in to osteoblasts producing cells. This work suggests that nHA from salmon fish bone is an excellent biomaterial candidate for bone tissue engineering applications.

2.2. Materials and methods

2.2.1. Preparation of salmon fish bone

Salmon fish bones were supplied from a local fish market, Busan, South Korea. Bones were cut in to smaller pieces using a wooden hammer and bladed cutter. Bones were boiled with 2 L of H_2O at 200 °C for 3h to remove the flesh. 1 Lof H_2O was added and boiled for 4h to remove all tissues remnants. Washed bones were collected and further boiled with 10 mL of acetone and 2% NaOH (10g/500ml of H_2O) for 1h to get rid of remaining tissues. The solution was continually flushed with H_2O to ensure tissue removal and was oven dried at 100 °C for 3h to remove moisture. The bones were crushed with a mortar and pestle.

2.2.2. Isolation of hydroxyapatite from salmon bone

nHA was isolated from salmon bone using alkaline hydrolysis (Jayachandran Venkatesan, Zhong Ji Qian, et al., 2011). Crushed bone (10g) was heated with 2M NaOH (Junsei Chemical Co., Ltd., Tokyo, Japan) for 1 h at 200 °C. This process was repeated until all traces of organic and collagenous materials were removed. nHA was collected in to conical tubes and centrifuged (Combi-514R, Hanil Science Industrial Co., Ltd., Incheon, Korea) at 1000rpm for 5mins, washed with H_2O until it reached neutral pH and dried in an oven at 100 °C.

2.3. General characterization

2.3.1 Thermogravimetric analysis

Thermogravimetric analyses of nHA were performed using a Pyris 1 TGA analyzer (Perkin-Elmer, TGA-7, Waltham, MA, USA; Model: TGA-7) with a scan range from 50°C to 700°C; constant heating rate of 10°C min^{-1} under continuous nitrogen.

2.3.2. Fourier transform infrared spectroscopy

Infrared spectrum resolution frequencies of the nHA were determined by Fourier Transform Infrared Spectroscopy (JASCO FT/IR-4100, JASCO, Tokyo, Japan) and a spectra manager (Serial number: C251761016) with a range of 400 to 4000 cm^{-1}.

2.3.3. X-ray diffraction analysis

The atomic and molecular structure of the nHA crystals were analyzed using X-ray diffractometer (PHILIPS X'pert, PANanalytical, Almelo, The Netherland); Cu-Kα radiation (1.5405Å) over a range of 5° to 80°, step size 0.02 and a scan speed 4° min^{-1} at 40kV and 30 mA were used.

2.3.4. Microscopic analyses

The morphology of the nHA crystals were characterized by Field-Emission Scanning Electron Microscopy (FESEM, JSM-6700F, JEOL, Tokyo, Japan)

and Transmission Electron Microscopy (HITACHIH-7500, Hitachi, Ltd. Tokyo, Japan).

2.3.5. Cell culture studies

MSCs were purchased from ATCC (American Tissue Culture Collection, Manassas, VA, USA), and were cultured in Dulbecco's Modified Eagle's Medium (BioWhittaker®, Madison, WI, USA) containing 10% Fetal Bovine Serum (FBS) (Serana® Bunbury, Australia) and 10ml Penicillin-Streptomycin (BioWhittaker®) in a 37 °C humidified atmosphere of 5% CO_2.

2.3.6. Cytotoxicity assessment

MSCs were cultured in cell culture dish 100×200 mm (SPL life sciences, Gyeonggi-do, Korea) in a humidified incubator (SANYO CO_2 Incubator-MCO-15AC, SANYO Electric Co., Ltd., Osaka, Japan) of 5% CO_2 at 37°C containing 10% FBS and 10ml antibodies (Penicillin-Streptomycin). Cells were harvested when confluent and then seeded in a 24-well plate containing 1mL medium at final density of 1×10^5 cells/mL. Concentrations (250 μg/mL, 100 μg/mL, 50 μg/mL, 10μg/mL and 0μg/mL (blank)) of isolated nHA were added to each plate. Cells were incubated for 24h. The media was removed and 1mL of MTT (0.0125mg/25ml) was added to each well and incubated for 4 h at 37°C. The MTT was removed and formazan crystals were stabilized by the addition of Dimethyl Sulfoxide (DMSO) (1mL/well). The MTT assay was quantified using GENios® microplate reader (Tecan Austria GmBH, Grödig, Austria) at an absorbance of 570 nm.

2.3.7. Optical microscopy

To examine the interaction of nHA and MSCs, cells were fixed with 2.5% glutaraldehyde (Sigma-Aldrich). Cells were examined using an optical microscope (CTR 600; Leica, Wetzlar, Germany).

2 The amount of mineralization produced was quantified by Alizarin Red-S (ARS) stain. DMEM medium, ODM media and nHA treated MSCs were incubated for 14 days. Cells were washed and treated with Alizarin Red-S

(Sigma-Aldrich) (0.2 g/20 mL of H_2O). The cells were fixed with 70% ethanol at room temperature for 1 h. The ethanol was removed, and 1 mL of Alizarin Red S (pH 4.2) was added for 15 min. Cells were washed with H_2O and the optical microscopy (CTR 6000; Leica, Wetzlar, Germany) images were taken. To quantify the minerals, 1 g of cetylpyridinium chloride (Wako Pure Chemical Industries Ltd., Osaka, Japan) was prepared with (0.2 g/20 mL of H_2O) sodium phosphate (Sigma-Aldrich). Each well was treated with 1 mL of the prepared concentration for 15 min. The optical density was determined by a microplate reader (Tecan Austria GmBH, Grödig, Austria) at 562 nm.

2.3.9. Statistical analysis

Statistical analyses were performed by Graphpad Prism 5. All experiments were run in triplicate, and the data were presented as the mean value ± standard deviation (SD) of each group.

2.4. Results and Discussion

2.4.1. General observations

Salmon fish are abundant in South Korea and are widely used as a food source. Salmon waste (scales, skin and bones) is discarded by local fish markets and industrial companies. This waste is hazardous to the ecosystem and represents an environmental and health risk. To avert this risk, we employed a cost efficient method of producing nHA from salmon bones for commercial and biomedical applications. Initially, raw salmon bone is covered by fish tissue and stored in a freezer. Crushed salmon bone is yellow in color. After alkali treatment, a light yellow color was observed. This color change indicates the removal of organic matter from the crushed bone.

2.4.2. Thermal gravimetric results of salmon HA

Thermal gravimetric analysis (50-700°C) of raw bone, crush bone and nHA-salmon are depicted in Figure 1. Two different points of weight loss (350 °C and 463 °C) were observed in the TGA spectrum of raw bone and crushed bone.

These temperatures correspond to the organic moieties. Alkaline treated crush bone demonstrate only one deflection at 465 °C, which may be attributed to the the small amount of the organic moieties present in the HA salmon bone.

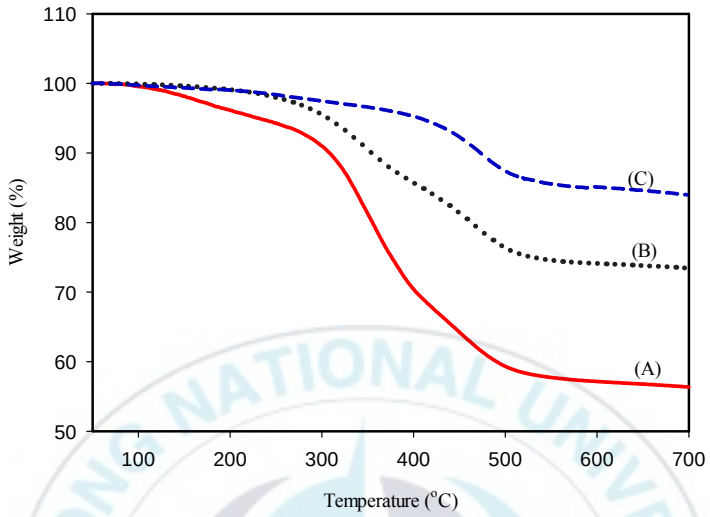

Figure 12. Thermal gravimetric analysis of (A) raw salmon bone (B) crushed s almon bone and (C) Nano-Hydroxyapatite salmon bone after alkaline treatment.

2.4.3. FT-IR spectra results

Fourier transform infrared spectroscopy (FT-IR) is a reliable reference technique to study the intra- and intermolecular interactions of a material. FT-IR was performed to identity the functional group of the isolated nHA. Figure 13(A) depicts the FT-IR spectrum of raw bone, crushed bone, nHA-salmon and synthetic HA from Sigma. The characteristic bands of raw bone were observed at 566, 601, 717, 1038, 1102, 1159, 1458, 1551, 1649, 1745, 2857, 2926, 3008 and 3431 cm^{-1}. These bands indicate calcium phosphate and collagen moieties. A strong band was observed at 1000–1100 cm^{-1}, indicating the stretching mode of PO_4 vibration. A band at 567 cm^{-1} corresponds to the n4 symmetric P–O stretching vibration of a PO_4 group. The band at approximately 3400 cm^{-1} corresponds to the O–H stretching of nHA. Figure

13 (B) represent the crushed bone IR spectra, 1745 cm^{-1} band was reduced, suggesting the removal of organic matter. However, the organic matter as indicated by the bands at 1450, 1569, 1646 and 1742 cm^{-1}. This bands corresponds to collagen.

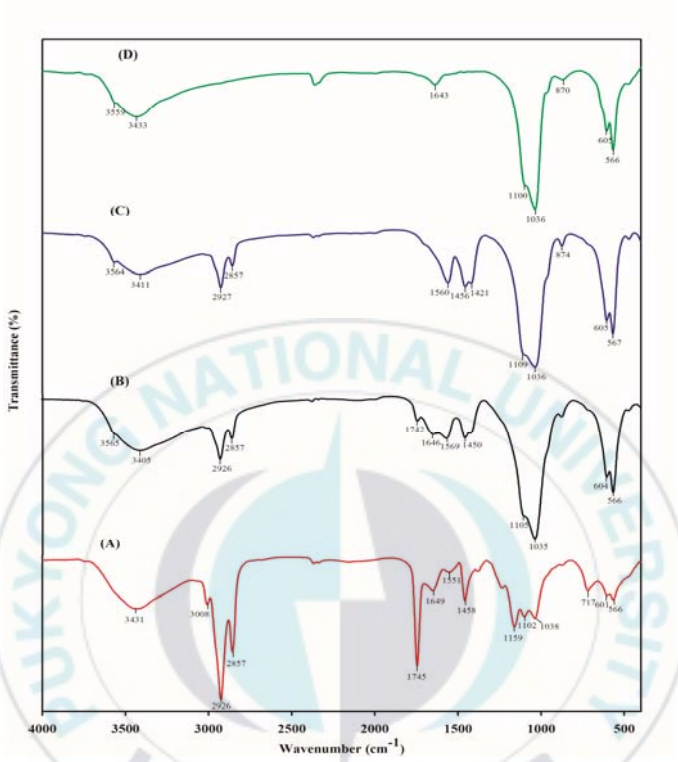

Figure 13. FT-IR spectra of (A) raw salmon bone (B) crushed salmon bone (C) nHA salmon bone after alkaline treatment and (D) HA-Sigma.

Figure 13 (C) represents the alkaline hydrolysis derived nHA. Significant differences were observed in the stretching frequencies of nHA salmon compared to raw bone and crushed bone. Several bands were present crushed bone were absent in nHA salmon, indicating the removal of organic matter from the crushed bone. The characteristic bands of nHA salmon are 567, 605, 874, 1036, 1109, 1421, 1456, 1560, 2857, 2827, 3411 and 3564 cm^{-1}. The bands of carbonated group (A and B type) are 1560, 1421 and 1456 cm^{-1}. Figure 13 (D) depicts the infrared spectrum of HA from Sigma-Aldrich (St. Louis, MO, USA). The characteristic bands are 566, 605, 870, 1036, 1100, 1643, 3433 and 3559

cm^{-1}. Difference were observed between HA sigma and nHA salmon. Carbonated groups are absent in the HA sigma.

2.4.4. X-Ray Diffraction results

A broad single peak was observed in the X-Ray diffraction spectrum of raw bone at 32.7, confirming that the nHA is amorphous. In Figure 14 (B&C), two peaks were observed at 32.1 (211) and 26.3 (002) for crushed bone; 31.9 (211) and 26.1 (002) for nHA salmon bone. The intensities of the peaks were higher in crushed and HA salmon bone compared to raw bone. The highest intensity of the peaks of nHA (31.9) is similar to that of the standard JCPDS 090432 (31.7). X-ray diffraction analysis (XRD) analysis suggests that the purity of the nHA salmon is higher than that of crushed bone.

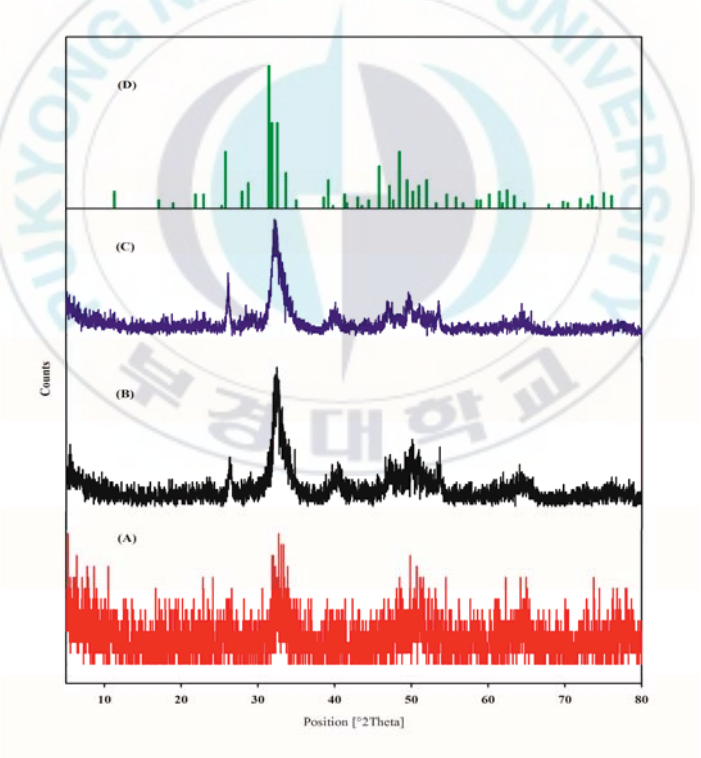

Figure 14. X-Ray diffraction spectra (A) raw salmon bone (B) crushed salmon bone (C) nHA salmon bone after alkaline treatment and (D) JCPDS 090432.

2.4.5. Microscopic results

Figure 15 depicts the FE-SEM images of nHA salmon at different

magnifications: (A) x 500, (B,C) x 1000 and (D) x 2500. Agglomeration of the nHA particles was observed. Figure 16 depicts the transmission electron microscopy images of nHA salmon for different scale bars, (A) 200 nm, (B) 100 nm and (C) 50 nm, and (D) selective area image diffraction. TEM analysis demonstrated that the crystal sizes were 6–37 nm with a nanorod shape. The nHA salmon particles were also analyzed using selective area diffraction analysis. The selective area diffraction results were consistent with the XRD results with the planes (002) and (211). These studies also confirmed that the alkaline treatment did not affect the crystal size of HA salmon.

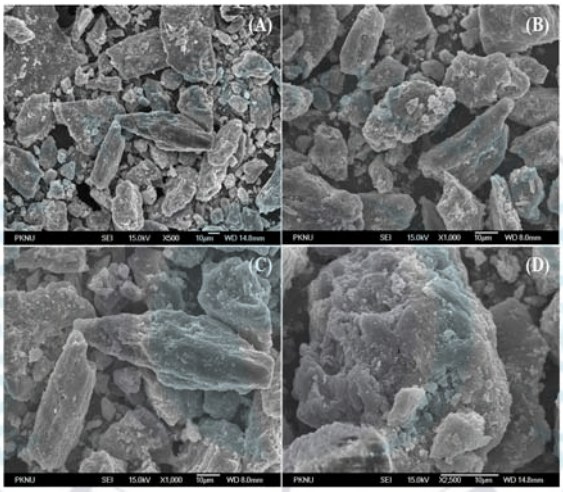

Figure 15. Field Emission Scanning Microscopy images of nHA salmon at dif ferent magnification (A) ×500; (B,C) ×1000 and (D) ×2500.

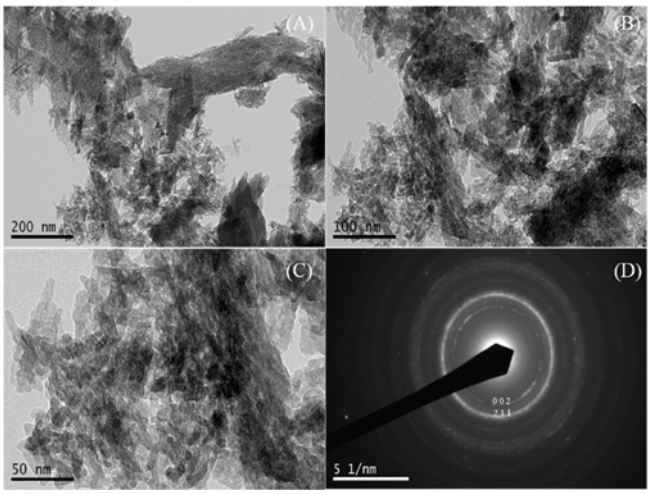

Figure 16. HR-TEM micrographs showing the appearance of the obtained

nHA crystals at different scale bar (A) 200 nm (B) 100 nm and (C) 50 nm from fish bones after alkali treatment (D) The corresponding selective area diffraction data of nHA.

2.4.6. Cell culture results

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to study the cytotoxicity of nHA salmon crystals to MSCs. Figure 6 reports the cytotoxicity results of nHA with MSCs at different concentrations (10, 50, 100 and 250 μg/mL). The results suggest that nHA crystals are not toxic to cells at 100 μg/ml. The cell morphology of nHA treated MSCs was determined using optical microscopy. The results indicated a slight inhibition of cells at concentrations higher than 250 μg/ml. However, the cells proliferated without any toxic effect at lower concentrations. The cell growth of the control was similar to that of the individually seeded lower concentrations. The cell growth of the control was similar to that of the individually seeded lower concentrations of nHA.

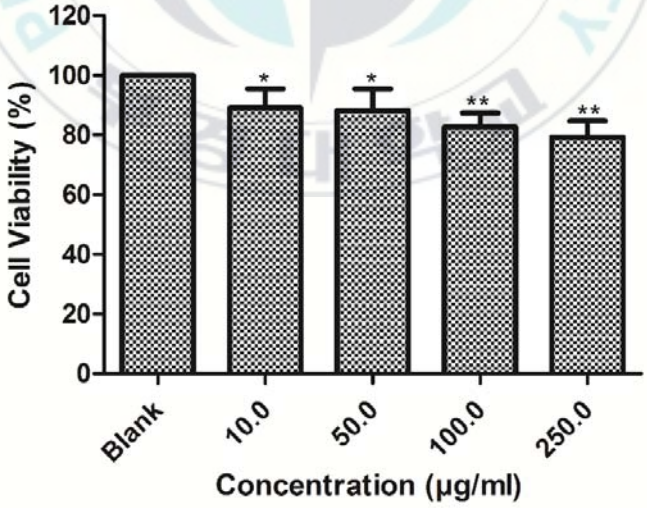

Figure 17. Cytotoxicity of nHA salmon crystals to mesenchymal stem cells at different concentrations. *: $P \leq 0.05$; **: $P \leq 0.01$.

2.4.7. Morphological studies with Optical microscopy

Morphologies of the MSCs with nHA treatment were studied under the optical microscopy as shown in Figure 7 (I). We observed that there is no known obstructions or modification in the morphologies of the MSCs, when interacted with nHA.

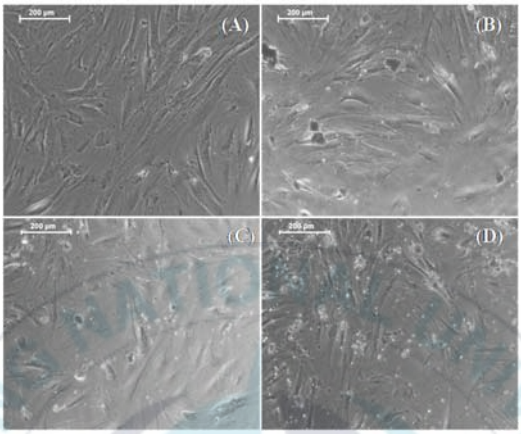

Figure 18. Phase contrast Optical Microscopy images of Mesenchymal Stem Cells with nHA at (A) blank (B) 50 µg/mL (C) 100 µg/mL and (D) 250 µg/mL

2.4.8. Mineralization results

Studying mineralization is an important as aspect in the development of advanced materials for use in organ regeneration. It also allows an understanding of how minerals can be produced by tissues (Palmer, Newcomb, Kaltz, Spoerke, & Stupp, 2008). The extracellular matrix is a highly organized nanocomposite, that models in cell function and biochemical processes. Tissue engineers use a nanotechnological approach to mimic this important matrix (Dvir et al., 2011). Binding sites exists in the form of nano-scaled fibers for cell adhesion. Nano-scaled protein fibers provide elasticity and strength . (Goldberg, Langer, & Jia, 2007). The most stable form of calcium phosphate is nHA. It forms 70% of the dynamic and highly vascularized bone tissue. Figure 19 depicts the mineralization effect of nHA with MSCs for (A) Dulbecco's Modified Eagle's Medium (DMEM), (B) ODM (Osteogenic differentiation medium), and (C&D) 100 µg/mL nHA salmon treated with

ODM media. These results the production of minerals by MSCs induced by the nHA salmon. Quantification of the minerals produced by the control, ODM and HA treatments showed relative mineralization of 100%, 104%, 118.26% respectively. This results implies that the HA salmon crystals can induce mineralization.

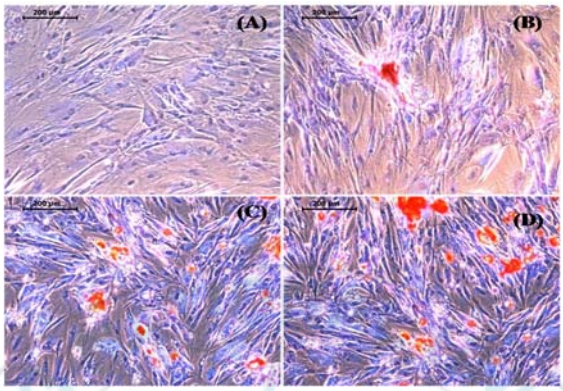

Figure 19. Alizarin red S stained images after 14 days of MSCs with nHA at 100 µg/ml concentration (A) Dulbecco's Modified Eagle's Medium (DMEM) (B) Osteogenic Differentiation Medium (C &D) 100µg/mL of nHA.

2.5. Conclusions

In our investigation we have isolated pure nHA from salmon fish bone using the alkaline hydrolysis method. This method described in the isolation of nHA is cost efficient. FT-IR results confirmed the presence of carbonated group (preferable in biomedical applications). XRD results, determined that the crystals are amorphous. Scanning electron and transmission electron microscopy results revealed that the crystals exhibit a nanostructure with a size range of 6-37 nm. Cytotoxity analysis of isolated nHA and MSCs suggest that the nHA is nontoxic and biocompatible. The nHA induced higher mineralization in MSCs, which is important for bone tissue engineering applications. The successful isolation and characterization of this important nano-material will be useful biomedical applications, especially in bone tissue engineering through its development. The development of this material via the processes highlighted in this paper may reduce the environmental effects of byproducts from the salmon

industry while efficiently safeguarding industrial pollution and waste management. This research suggest that nHA salmon is an alternative biomaterial with potential for biomedical applications in the field bone tissue engineering.

Chapter 3.

Preparation and characterization of chitosan-natural nano hydroxyapatite-fucoidan nanocomposite for bone tissue engineering

In this chapter, a solid three dimensional (3D) composite scaffold with a high potential usage for bone tissue engineering was prepared using freeze drying method composed of chitosan (C), natural nano-hydroxyapatite (nHA) and fucoidan (F), (C-nHA-F). Fourier transform infrared spectroscopy (FT-IR), Thermal Gravimetric Analysis (TGA), X-ray Diffraction Analysis (XRD), Scanning Electron Microscopy (SEM) and Optical Microscopy (OM) were used to determine physiochemical constituents and morphology of the scaffold. The addition of nHA in the C-F composite scaffold reduce the water uptake and water retention ability. FT-IR analysis shows the presence of carbonated group in the scaffold which is due the presence of nHA that was isolated via alkaline hydrolysis from salmon fish bones. Microscopic results indicate that dispersion of nHA and fucoidan in the chitosan matrix were uniform and shows the pore size (10 to 400 μm) of the composite revealing a suitable micro architecture for cell growth and nutrient supplements. This was further elucidated *in vitro* using Periosteum-derived Mesenchymal Stem cells (PMSCs) and revealed profound biocompatibility and excellent mineralization. Thus, we suggest that Chitosan-nHA-Fucoidan composite will be a promising biomaterial for bone tissue regeneration.

3.1. Introduction

Tissue engineering is an interdisciplinary research to constructing the

artificial organs using materials, cells and growth factors. Bone is an important organ which remodels continually during an individual's life time and forms the human skeletal framework. Bone tissue engineering is an important aspect of regenerative/nanomedicine and biotechnology providing alternatives therapeutic methods in restoring the function of and or repair damage or degenerating bone tissues. Biomaterials with functional subunits which provide advantages that promote bone tissue regeneration and facilitate the repair of damage bone tissues, thus, they have huge potential applications in clinical orthopedics during reconstructive surgery procedures. Autologous transplantation presents best effect in clinical surgery procedure as it helps to effectively unite the host bone tissue without immunogenic complications and those related to disease risks from allogeneic sources. Although effective, it's highly limited in supply driven by the rise in medical and socioeconomic constraints of the world's aging populations. This is also due to the fact orthopedic constructions in trauma, tumor, congenital deformities and injuries from accidents are in record numbers requiring much demand in bone regeneration or implant technologies(Wu et al., 2014). Tissue engineers seeks to develop functional materials for this purpose which allows the delivery of nanoscale topographical bio factors cues of the extracellular matrix of cells. Understanding the biological effects of these materials is a fundamental requirement in tissue engineering as it further affirms their suitability and elucidate the role they play in tissue formation (Bauer & Muschler, 2000; Stevens, 2008). In bone tissue engineering substitutes are engineered in the form of scaffolds to ensure that they are non-toxic and biocompatible allowing controllable biodegradability and oestoconductive(Sai Zhang, Prabhakaran, Qin, & Ramakrishna, 2015a). To ensure this, various fabrication techniques are employed to satisfy these important requirements. Techniques such as freeze drying, particle leaching, electrospinning, soft lithography, 3D printing and photolithography can produce suitable micro and nano architecture with pivotal influence in stem cell adhesion, expansion, alignment, proliferation,

maintaining uniform shapes and enhancing their differentiation potentials(Chaubey, Ross, Leadbetter, & Burg, 2008; Chua et al., 2006; Kingsley, Ranjan, Dasgupta, & Saha, 2013; Ma et al., 2008). Nanotechnology can capacitate the model and fabrication of these scaffolds by providing the optimal mechanical properties for medical implantation and delivery as well control the spatiotemporal release of growth factor which can modulate the extracellular matrix by guiding cell behavior towards the generation of implantable tissues(Shi, Votruba, Farokhzad, & Langer, 2010).

Chitosan is a polysaccharide which makes us the exoskeleton of crustaceans and have a straight chain polysaccharide group composed of ß-(1-4)-linked D-glucosamine and N-acetyl glucosamine subunits(Jayachandran Venkatesan, Lowe, Pallela, & Kim, 2014) have been studied for various biomedical applications either itself or when functionalized with other materials. It is a partially deacetylated derivative of chitin readily soluble in dilute acids (pH<6). Chitosan is biodegradable, biocompatible, non-toxic and have a functional hydrophilic surface which promotes cells growth by modeling in adhesion, proliferation and differentiation and elicit nominal immunogenicity if use as an implant (Lou et al., 2014; Jayachandran Venkatesan & Kim, 2014; Jayachandran Venkatesan, Lowe, et al., 2014). Chitosan is suitable cross-linker which also acts as substrate biomaterial can mimic the extracellular matrix glycosaminoglycan thus enhancing cells adhesion, survival and proliferation. And with the required growth factors delivered chitosan can favorably serve as niche for stem cell differentiation in to desired lineages (Debnath et al., 2015). Several researcher have reported the use of the chitosan and synthetic nHA and natural nHA for bone tissue engineering application. Several researcher have reported chitosan- nHA for bone tissue engineering (Cai et al., 2009; Z. Li et al., 2005; Thein-Han & Misra, 2009; J. Venkatesan, Qian, Ryu, Ashok Kumar, & Kim, 2011; Y. Zhang et al., 2008).

nHA is natural mineral form of calcium apatite which chemically resembles

the complex matrix of bone compose of a hydroxyl and complex apatite denoted with the general formula $Ca_{10}(OH)_2(PO_4)_6$. Due to the nature of natural bone, its reconstruction require a formidable mechanical strength and nHA which is a constituent (about 70%) of bone mineral is used to build structural rigidity of scaffolds to make them suitable for use in bone tissue construction procedures. Because of the chemical similarities of the nHA to natural bone, their is extensive research towards the preparation of bone substitutes using nHA for biomedical application(H. Zhou & Lee, 2011). nHA is highly osteoconductive with bone bonding capacity and offers an appropriate template structure in bone formation processes and as well promote cellular functioning allowing the expression of bone forming osteogenic markers (LeGeros, 2002). Despites these remarkable properties, nHA is brittle and limits its direct application in loading bearing applications. Because of this nHA is functionalized with other polymers like chitosan to provide the mechanical properties required to ensure their effective usage as implant in reconstruction and regeneration of bone tissues (Wei & Ma, 2004). nHA can be isolated from nature sources such as bovine (Mangano, Scarano, Perrotti, Iezzi, & Piattelli, 2006), fish bone (Jayachandran Venkatesan, Baboucarr Lowe, Panchanathan Manivasagan, et al., 2015) and fish scales (Y.-C. Huang, Hsiao, & Chai, 2011). Certain religious sentiments limited the use of bovine sourced HA(Piccirillo, Silva, Pullar, da Cruz, et al., 2013).

Fucoidan is made up of a primary linear chain of $1 \rightarrow 3$ linkage of α-L-fucopyranose and other saccharide units made of xylose, galactose, mannose and glucuronic acid (Ponce, Pujol, Damonte, Flores, & Stortz, 2003; Jayachandran Venkatesan, Baboucarr Lowe, Sukumaran Anil, et al., 2015) including α–D-glucopyranosyluronic acid and α –L-fucopyranosyl residues can be found in some species such as *Cladosiphon okamuranus and Chorda filum respectively.* The sulfate ester and fucose groups of fucoidan are essential units with functional cellular activities targeted for use of this anionic polysaccharide for tissue engineering. It triggers biological activities of the alkaline phosphatase (ALP) and osteocalcin (OC) which are phenotypic markers

in early stages of osteoblast differentiation and as well enhance febrile collagen matrix formation thus resulting in stimulation both *in vitro* and *in vivo* angiogenesis (Cho, Jung, Kim, Choi, & Kim, 2009; Jeong, Venkatesan, & Kim, 2013). Fucoidan can also induce important osteogenic genes such as bone morphogenetic protein-2 (BMP-2), Collagen-1, Osteocalcin which are highly essential to construct the artificial bone improving bone cell adhesion and proliferation.

Our hypothesis is, addition of fucoidan in the presence of functional carbonated nHA in chitosan would be an excellent biomaterial composite to bone repair and or regeneration. To examine the functional role of these biomaterials and elucidate it's biological activities for bone tissue engineering. We investigated and fabricated a 3D scaffold using a freeze-drying method and functionalized nHA from salmon with chitosan and fucoidan for its role in stem cell differentiation of periosteum derived-mesenchymal stem cells (pMSCs) for use a potential composite bone tissue engineering applications. Here, we report for the targeted differentiation in PMSCS using nHA and fucoidan as a functional polymers. Further, hypothetically, the functional subunits present in the fucoidan (fucose and sulfate ester), nHA derived from salmon (carbonated groups) and hydrophilic units of the chitosan will affect bone mineral formation producing osteoblastic lineages from pMSCs.

3.2. Materials and Methods

Chitosan (310kDa) of 90% deacetylation purchased from Kitto Life Co., South Korea. Fucoidan from *Fucus vesiculosus* was acquired from Sigma-Aldrich Co., (St. Louis, MO, USA). Natural nHA were isolated as per our previous published paper(Jayachandran Venkatesan, Baboucarr Lowe, Panchanathan Manivasagan, et al., 2015). Acetic acid was obtained from Junsei Chemical Co., Ltd. Stem cell expansion medium (SCEM) was obtained from BioWhittaker®, (Walkersville, MD, USA). MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide was obtained from Sigma, (St. Louis, MO, USA). Perisoteum mesenchymal stem cells (PMSCs) were obtained from the

American Type Culture Collection (Manassas, VA, USA). All other reagents employed in this experiments were of analytical grade.

3.3. Fabrication procedure of scaffolds

3.3.1. Preparation of chitosan scaffold

2.5 % chitosan solution was stirred at 1200 rpm overnight. 5 to 6g of solution was transferred in to a petri dish and freeze at -24°C. It was lyophilized using a freeze dryer. The scaffold were finally regenerated with 10% NaOH and flushed with excess water to obtain a neutral pH of 7 and were further lyophilized as represented in **(Figure 20).**

3.3.2. Chitosan-Fucoidan Scaffold

To prepare chitosan-fucoidan scaffold, 0.1g of weight fucoidan (*Fucus vesiculosus*-Sigma) was first dissolved in small amount of H_2O and finally added drop wise in to a beaker containing 2.5% chitosan solution. This was allowed stir overnight at 500rpm. The scaffold was prepared following the same protocol highlighted above.

3.3.3. Chitosan-natural nano hydroxyapatite Scaffold

2.5g of natural nHA derived from salmon was used and added to 2.5% chitosan solution. It was further stirred to ensure a homogeneous mixture and was subsequently lyophilized and regenerated using the same method.

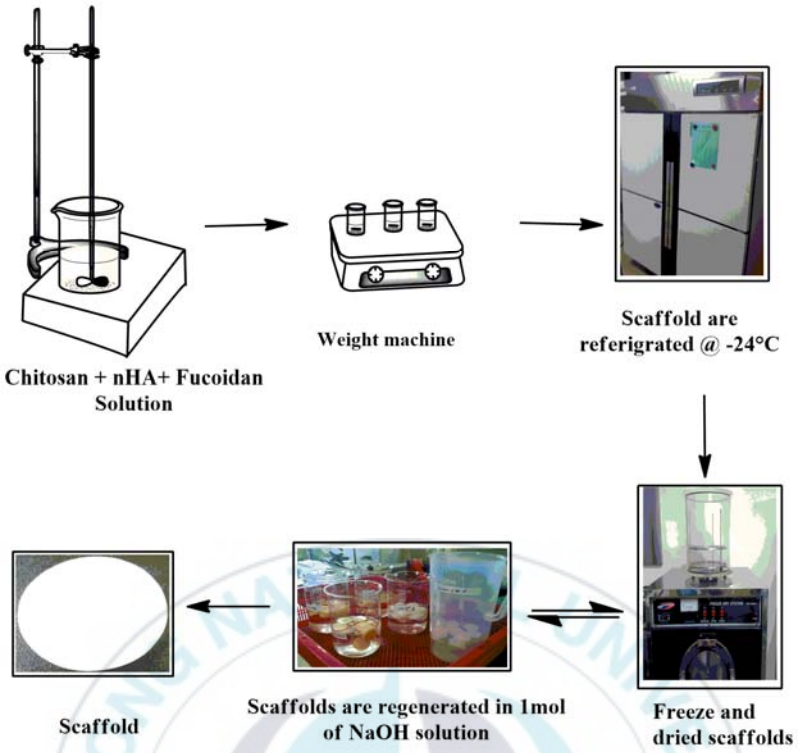

Figure 20. Schematic representation of the fabrication method of chitosan-nHA-fucoidan composites.

3.3.4. Chitosan- natural nHA-Fucoidan Scaffold:

The ratio of chitosan and fucoidan were maintained as previous with chitosan-fucoidan scaffold and 2.5g of nHA from salmon added to the mixture and stirred at a lower rpm. Finally the scaffold was fabricated using the same procedure.

3.4. Experimental Section

3.4.1. Thermogravimetric analysis

Pyris 1 TGA analyzer (Perkin-Elmer, USA; Model: TGA-7) was used to carry out Thermogravimetric and Derivative Thermogravimetric (TGA-DTG) analyses at scan range from 50°C to 800°C; constant heating rate of 10°C min-1 under continuous nitrogen flow.

3.4.2. Fourier transform infrared spectroscopy

Infrared spectrum resolution frequencies of the sample were determined by Fourier Transform infrared spectroscopy (JASCO FT/IR-4100; Tokyo, Japan) and spectra manager (Serial number: C251761016) within the range of 400 to 4000 cm^{-1}.

3.4.3. X-ray diffraction analysis

Using X-ray diffractometer (PHILIPS X'pert MPD diffractometer, Netherland); Cu-Kα radiation (1.5405Å) over a range of 5° to 80°, step size 0.02, scan speed 4° min-1 at 40kV and 30 mA the atomic and molecular structure of the sample crystals were analyzed.

3.4.4. Porosity

The porosity of the scaffolds was measured using the liquid displacement method as reported by (Jayachandran Venkatesan, Bhatnagar, & Kim, 2014) in ethyl alcohol. Briefly, the weight of the scaffold was taken and immersed in a beaker containing the ethyl alcohol and left for 24 hr. until saturation. The scaffolds were weight again. The porosity was finally calculated using the formula below.

$$\text{Porosity} = (V_2 - V_1 - V_3)/ (V_2 - V_3)\ X100$$

3.4.5. Water uptake and retention

This was studied using the method previously reported by (Pallela, Venkatesan, Janapala, & Kim, 2012). To measure this, the scaffolds were weight (W_{dry}) and immersed in distilled water for 24 h. The scaffolds were gently removed and laid on a wire mesh of tissue paper to drain excess water and further weight to determine the water absorption levels. To evaluate the water retention capacity of the scaffold, the scaffolds were place in a centrifuge tube and centrifuged (Combi-514R, Hanil Science Industrial Co., Ltd., Incheon, Korea) at 500 rpm for 3 min and weight again (W'_{wet}). Water uptake and retention was then calculated using the formula below.

EA= [Wwet – Wdry/Wdry] X 100

ER= [W'wet – Wdry/Wdry] X 100

3.4.6. Scanning Electron Microscopy

The morphology of the scaffolds were characterized by Field-Emission Scanning Electron Microscopy (FESEM, JSM-6700F, JEOL, Japan)

3.4.7. Optical microscopy

Scaffold structural network and integrity and pore size were examine via optical microscopy. With the aid of a blade, the scaffolds were resized (10mm length and 2mm width) to further validate the internal structure view; this was observed with an optical microscope (CTR 600; Leica, Wetzlar, Germany). The pore surface structure was uniform showing similar pore sizes of similar length.

3.4.8. PMSC cell culture

Periosteum mesenchymal stem cells (pMSCs) were expanded in stem cell expansion (SCE) medium in T-75 flask cell culture dish and incubated in a humidified incubator (SANYO CO2 Incubator-MCO-15AC, SANYO Electric Co., Ltd., Osaka, Japan) of 5% CO2 at 37°C containing 10% Fetal Bovine Serum (FBS) and 10mL antibodies (Penicillin-Streptomycin). Upon reaching confluence the cells were trypsinized and carefully harvested and seeded (5X104 cell/ml) in to the tissue culture dish 100 × 200 mm (SPL life sciences, Gyeonggi-do, Korea) containing samples for use in experimental analyses.

3.4.9. Cytotoxicity assessment

This was carried out using the cell viability assay- MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium). First, the cells were cultured and upon reaching confluences trypsinised and harvested. Further, $5X10^4$ cell/ml was added to each well containing the scaffold. Earlier, the scaffolds were made to fit individual wells of the microplate; using a sharp cutter it was resize about 2mm in diameter and thickness of 1.5mm. It was later sterilized in 100%, 70% and 50% ethanol for 1hr, 30min and 30min

respectively and further washed 5 times in 1X PBS. 1ml of stem cell expansion (SCE) medium containing 10% FBS and 1% antibodies (Penicillin-Streptomycin) was added to each well containing the scaffolds and incubated in a humidified condition (SANYO CO_2 Incubator-MCO-15AC) of 5% CO_2 at 37°C for 48hr. Prior to seeding the cells the medium was removed and scaffolds washed again with fresh SCE medium. Finally the cells were seeded and incubated for 24hr and 48hr for evaluation. To evaluate cell viability, the MTT was used. Briefly, medium was removed and 1ml of MTT (0.0125mg/25ml)-(20% of MTT in 50ml DMEM FBS^-) was added to each well and incubated for 4h under same condition mentioned above. The MTT was aspirated and 1ml of DMSO (Dimethyl sulfoxide) was added to each well to dissolve the formazan crystals. Further the absorbance was quantified using a GENios® Microplate reader (Tecan, Austria GmBH) at 570nm wavelength.

3.4.10. Optical Microscopy of scaffolds with cells

To examine the cell growth on the scaffold matrices, the scaffolds were resized in to thin films and carefully sterilized in UV (10mins) and washed with 100% for 1 h and later 70%, 50% ethanol for 30 min. Further, they were incubated under the same culture condition with SCE medium only for 48 hr to ensure that there is no alcoholic residue left on the scaffolds. After, $5X10^4$ cell/ml was seeded in to the scaffolds and cultured for 14 and 18 days and later fixed and observed using an optical microscope (CTR 600; Leica, Wetzlar, Germany) to determined cell adhesion on the scaffold matrices. Results of the assessment as shown in Figure 6 (I) and (II) revealed exclusive cell adhesion on the scaffolds of C, C-F, C-nHA, and C- nHA-F. This further confirmed the no-toxic nature of the scaffolds. The level of cell proliferation also showed the role of the functional subunits present as the cellular matrix surface reaction with the scaffolds was a well suited environment promoting the stem cell growth and differentiation.

3.4.11. Mineralization study

To account for osteoblast progenitors and production of bone minerals of the

differentiated PMSCs. The cells were treated with ODM (osteogenic differentiation medium) for 28 days. Which was followed by mineral quantification via alizarin Red-S assay. To confirm and quantify produced minerals, the medium was removed and scaffolds were rinse twice with 1XPBS and fixed with 70% ethanol at room temperature for 1hr. Further, they were stained with 40mM Alizarin Red S (pH 4.2) and left for 10 minutes upon which the cells were wash with deionized water and PBS several times. The scaffolds were further treated with 10% of cetyl pyridium chloride in 10mM of Sodium phosphate (pH 7.0) buffer for 15 minute and the optical density was quantified with a microplate reader (Tecan Austria, GmBH) at 562nm.

3.5. Results and Discussion

3.5.1. General observation

The fabricated scaffolds by nature have a round 3D structural framework of diameter 1.3cm and 0.5cm width/ thickness with a respectable degree of stiffness**.** The functionalized composite scaffolds are made up of chitosan, fucoidan and nHA isolated from the bones of salmon. Chitosan and Chitosan-F scaffolds are pale yellowish in color with C-nHA (Chitosan-nHA) C-nHA-F appearing to having a slight white-yellow coloration. This is due to the dispersion of the fucoidan and nHA in the chitosan solution as shown in (**Figure 21**).

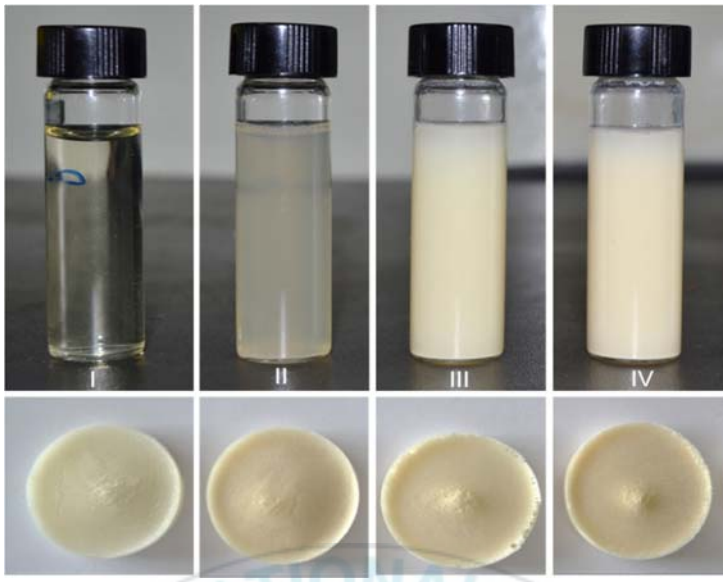

Figure 21. (I) chitosan, (II) chitosan-fucoidan, (III) chitosan-nHA and (IV) chitosan-nHA-Fucoidan solution and their respective composite scaffold after alkali regeneration.

3.5.2. Porosity of the scaffolds

The porosity was measured by a liquid displacement method using ethyl alcohol. C, C-F, C-nHA, C-nHA-F have recorded porosity levels of 97%, 97%, 95% and 92% respectively. This suitable and place an edge application in tissue engineering. The difference in porosity levels observed might have been a result of the nHA which is present in the C-nHA and C-nHA-F scaffolds. The porosity of the scaffold to be implanted is essential factor as it plays a significant role in biological delivery and effectively function in mass transport thus optimizing tissue engineering treatments (Hollister, 2005). This further gives a comfort zone for cells to grow in a normal morphological architecture and enhance vascularization of the ingrown tissue. A typical porosity of 90% is a fundamental requirement for cell penetration as well to ensure the right vascularization of ingrowing tissues(Griffith, 2002; Griffith & Naughton, 2002; Karageorgiou & Kaplan, 2005; Mikos & Temenoff, 2000; Rezwan, Chen, Blaker, & Boccaccini, 2006)

3.5.3. Water Absorption and Retention

The water absorption capacity of the scaffolds were assessed to examine their suitability in bone tissue damage repair and or regeneration applications. Results in **Figure 22 (I)** shows that all the scaffolds exhibited similar absorption rates but still a disproportionate difference in retention capacities were observed with C-nHA, C-nHA-F bearing less compared to C and C-F scaffolds. Nevertheless, it proves efficient for use in tissue regeneration purpose in terms of percentage per volume ratio. Although the absorption rate was sufficient. It also suggest that rate of absorption exhibited by the scaffold was much less rapid but tolerable comparatively and this might be due to the dense polymeric presence of the nHA in the C-nHA, C-nHA-F scaffolds. The water retention of the C-nHA, C-nHA-F was less compared to C and C-F this is linked to the presence of the nHA which is incredibly less restorable (Murugan & Ramakrishna, 2004).

3.5.4. Thermogravimetric Analysis results

In thermogravimetric analysis, weight changes were with increasing temperature on the physical and chemical properties of the scaffolds by the TGA-DTG results (Data not shown). In the C-F scaffold two points of deflection were observed indicating a weight loss at 100°C and 350°C. Similar observations were made on the C-nHA, C-nHA-F. A slight weight loss is visible on the C-nHA-F at 450°C which is also observe in the C-nHA. The initial deflection is corresponds to the presence of water moieties in the scaffolds. The second deflection is corresponding to the chitosan weight loss.

3.5.5. FT-IR Analysis Results

From the spectrum in **Figure 22 (III) (a)** the IR spectrum of the chitosan scaffold shows the distinctive absorption band at 3444 cm^{-1} which is as a result of the OH and amine N-H symmetrical stretching vibrations. The symmetric stretch of the $–CH_2$ appears at 2913 cm^{-1} and the absorption band of the (amide

I), (–NH_2) and (amide III) bending are showed at 1645 cm^{-1}, 1599 cm^{-1} and 1379 cm^{-1} respectively. Meanwhile, the skeletal vibration of the C-O stretch appears at 1078 cm^{-1} which is a feature of its saccharide framework or structure. Fucoidan has a broad band of O-H group stretching at 3450 $cm^{-1,}$ and C-H stretch assigned to the methyl and pyranose ring at 2940 cm^{-1} and 2885 cm^{-1} with other distinct sharp bands at 1600 cm^{-1} and 1420 cm^{-1} corresponding to the (COOH stretch). Further, with a (S=O) at 1250 cm^{-1} with couple of tiny bands at 840 cm^{-1} and 820 cm^{-1} attributed to the axial and equatorial C-O-S flexing (Y.-C. Huang & Li, 2014; Saboural et al., 2014). This is not remarkably visible in the C-F spectrum in Figure **22 (III) (b)** owing to the fact that a small amount of the functional polysaccharide was added in to the reaction mixture during composite fabrication. But a sharp projecting band observed between 2360 cm^{-1} and 1643 cm^{-1} which is clearly absent in the chitosan (CH) scaffold and the series of the numerical difference in the bands of the CH might be ascribed to the least present. Figure **22 (III) (d)** depicts the IR spectrum of the functionalized nHA isolated from salmon fish bone via an alkaline hydrolysis method as reported in (Jayachandran Venkatesan, Baboucarr Lowe, Panchanathan Manivasagan, et al., 2015). The stretching frequencies of the OH can be observed from 3564 cm^{-1} to 2927 cm^{-1} and those of the carbonated groups which is unique to naturally derived nHA can be observed at bands 1560, 1456 and 1421 cm^{-1}. The extending bands are ascribed to the bending modes of the P-O bonds in the phosphate groups which is also elucidated in Figure **22 (III) (c).** Carbonated nHA has essential biological properties which enhance tissue regeneration *in vitro and in vivo*.

Carbonated nHA show much higher biocompatible, bioresorpable and osteoconductive than synthetic nHA (Landi et al., 2003; Orr et al., 2001; Jayachandran Venkatesan & Kim, 2012).

Also Boilila et al elucidated the role of carbonated nHA in detoxification of rats exposed to nickel by reducing the level of lipid peroxidation makers and

increase defense enzyme activities (Boulila, Elfeki, Oudadesse, & Elfeki, 2015).

3.5.6. X-Ray Diffraction Results

Figure 22 (II) shows the X-ray diffraction results of the composites. In Figure **22** (II) (A and B) dense peak of the diffraction pattern at 11.0 and 20.0 which is slightly different from diffraction pattern of native chitosan (2θ = 10.6 and 19.9 degrees). This confirms the presence of the functional subunits of chitosan assemblage present in the C-F scaffold. In figure **22** (II) (C and B) two peaks are visible at 32.1(211) and 26.3 (002) this confirms the presence of the nHA in the composite and the small peaks shows the polymeric morphological subunit of the chitosan and fucoidan constituents present in the scaffold.

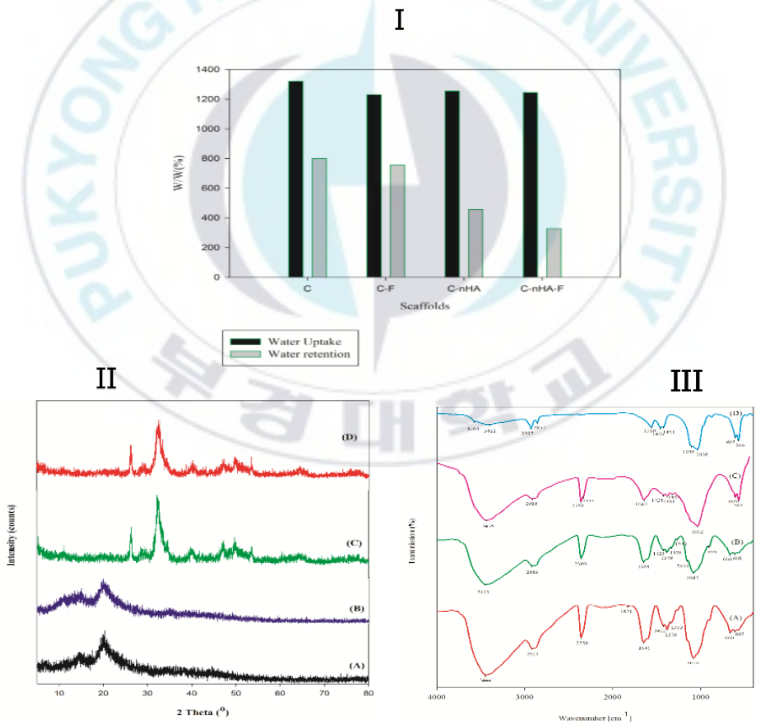

Figure 22. (I) shows the water absorption and retention levels of the scaffolds; 3 (II) shows results of X-ray diffraction analysis of (A) C (B) C-F (C) C-nHA (D) C-nHA-F scaffolds. (III) shows the FT-IR results of (A) C (B) C-F (C) C-nHA-F scaffolds and (D) nHA isolated from salmon fish bone.

3.5.7. Scanning electron microscopy and Optical microscopy

The optical microscopy and scanning electron microscopy results in **Figure 23 (I) and (II)** revealed the internal framework of the nanocomposite, and distribution of the pores. The optical microscopy results infers that the distribution of nHA in the chitosan and fucoidan matrix is uniform. There is no agglomeration observed and this is confirmed by optical microscopy. The incident light of optical microscopy is passed easily in the chitosan scaffold, where it was inhibited by chitosan-nHA and chitosan-nHA-F scaffold. This is indirectly tells about the uniform presence of nHA in the matrix.

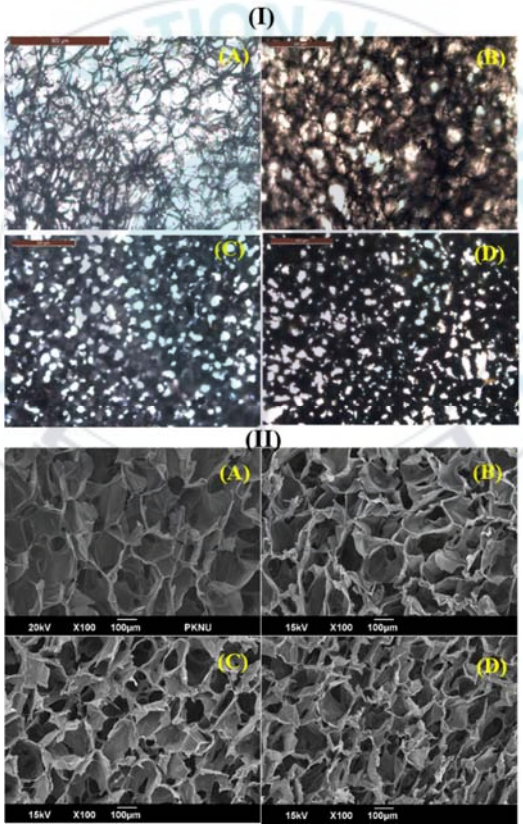

Figure 23. (I) optical microscopy images of (A) C (B) C-F (C) C-nHA (D) C-nHA-F scaffolds (II) Scanning Electron micrograph of the (A) C (B) C-F (C) C-nHA (D) C-nHA-F scaffolds, showing the pore size and internal network of the scaffolds.

Scanning electron microscopy were used to find the surface morphology and pore size of the scaffold. The porosity of the scaffold were found to be greater than 90% which was confirmed porosity measurement. The minimum and maximum pore size of the C-F, C-nHA, and C-nHA-F scaffolds were determined to be 14 to 332 µm, 10-263 µm and 23- 354 µm respectively. This pore is highly suitable of nutrient and blood supplements.

3.5.8. Cytotoxicity assessment

This is one of the most essential analysis to determine a biocompatibility of scaffolds for suitable application in tissue engineering and or use as a bone substitute. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium) is reduced by metabolically active cells, by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH.

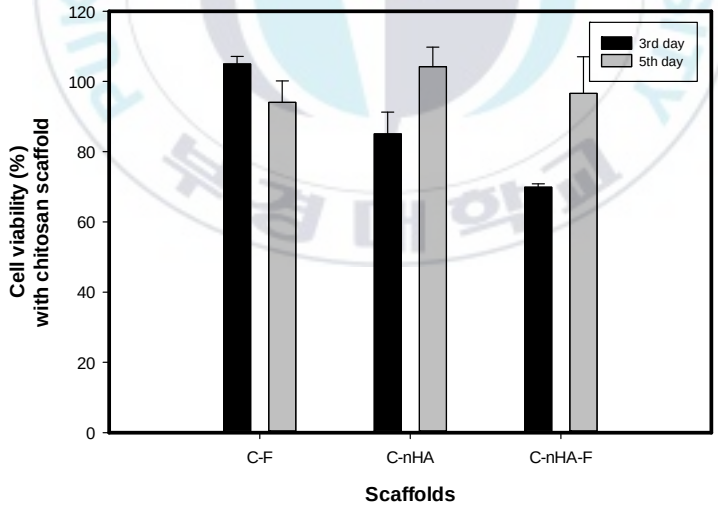

Figure 24. Cytotoxity assessment measured via MTT assay of the scaffolds in 3rd and 5th days of culture.

It elucidates cell viability by the activity of functional mitochondrion to oxidize the MTT and reduce the tetrazolium salts to formazan crystals (Haifeng Liu et al., 2004). Metabolic events which lead to apoptosis or necrosis cause reduction

of cell viability. It is thus use to measure cell proliferation. Periosteum derived mesenchymal stem cells were interacted with the scaffolds. The cell viability was maintain and was no toxicity as confirmed in results of the cytotoxicity assessment in **Figure 24** showed in C-F, C-nHA and C-nHA-F. This was further confirmed from results of the optical microscopy and the optical mineral density produced by the cells at 14 and 28 days as showed in **Figure 25 (I) and (II)** respectively which shows the cells growing on the scaffold matrices. A slight decrease was observed in C-F this might perhaps be a result a stress response, despite cell viability was more than 90% confirming that the C-F scaffold was not toxic and such response was not observed in C-nHA-F. Although fucoidan have been reported to promote human alveolar bone marrow-derived stem cells (HABM-MSC) it highly dose dependent for eliciting cellular response and bioactivity (Beom Su Kim, Kang, Park, & Lee, 2015).

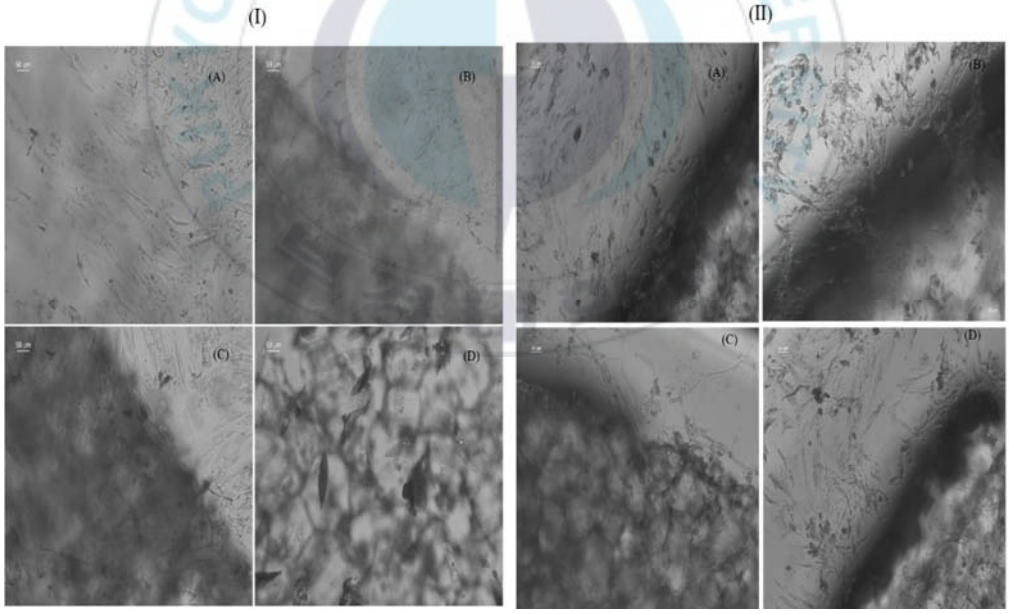

Figure 25. (I) Optical microscopy assessment of 14th day cultured Periosteum Mesenchymal stem cells on (A) C (B) C-F (C) C-nHA (D) C-nHA-F scaffolds (II) Optical microscopy assessment of 28th day cultured Periosteum Mesenchymal stem cells on (A) C (B) C-F (C) C-nHA (D) C-nHA-F scaffolds

Meanwhile, it is prudent to note that stem cells have recurring ability to reducing

stress and facilitate long term maintenance by functional activity of FOXO transcription factor function (Tower, 2012). Also, epigenetic changes in stem cells are not permanent and can be erase by cell division. This might facilitate self-renewal or prompt differentiation with diminish renewal capacity (Murray et al., 2013; Pazhanisamy, 2009). The increase proliferation witness might have been induced by the presence of the carbonated nHA present in C-nHA and C-nHA-F. Meanwhile, nHA from nature contains carbonated group which induce proliferation and enhance biocompatibility(Pallela et al., 2012) Such role of carbonated nHA promoting stem cell proliferation have also been observed and reported in our previous studies(Jayachandran Venkatesan, Baboucarr Lowe, Panchanathan Manivasagan, et al., 2015). Chitosan is non-toxic and biocompatible provides a conducive environment for cell ingrowth, osteoconduction, biodegradable, bio absorbable and its intrinsic antibacterial activity. For this, it a can be readily functionalized with other polymers to increase bioactivity for specific therapeutic applications(Di Martino, Sittinger, & Risbud, 2005; Q. Hu, Li, Wang, & Shen, 2004; Madihally & Matthew, 1999; Ueno, Mori, & Fujinaga, 2001; J. Venkatesan & S.-K. Kim, 2010).

3.5.9. Mineralization Results

Results of the mineralization assay in **Figure 26** indicated the production of minerals resulting from the differentiated stem cells. The inducing factors have been much associated with fucoidan and nHA present as evident our previous studies and by other researchers. Each scaffold has a functional polymer which affects mineralization *in vitro* as evident in other studies(S.-K. Kim & Cho, 2009; Rodriguez et al., 2012). Fucoidan is one of sulfated polysaccharide that have positive response in promoting proliferation of stem cells and osteoblast cell lines and it's bioactivity has been extensively studied(Cho et al., 2009; Jang et al., 2007; H. Kim, Lee, Jung, & Jeon, 2015; Park, Lee, Lim, & Lee, 2011). This is also reported in nHA (Jayachandran Venkatesan, Baboucarr Lowe, Panchanathan Manivasagan, et al., 2015) as a inducing factor mineral

production in adipose stem cells (Y. L. Chang, Stanford, & Keller, 2000). In our investigation the mineralization was two times higher in C-nHA-F scaffold compared to Chitosan and C-F scaffold and thus suggest that addition of the fucoidan further induce mineral production. This is evident by the present of the carbonated group in nHA which is highly desirable as it promotes oestoconduction *in vitro* and highly biocompatible.

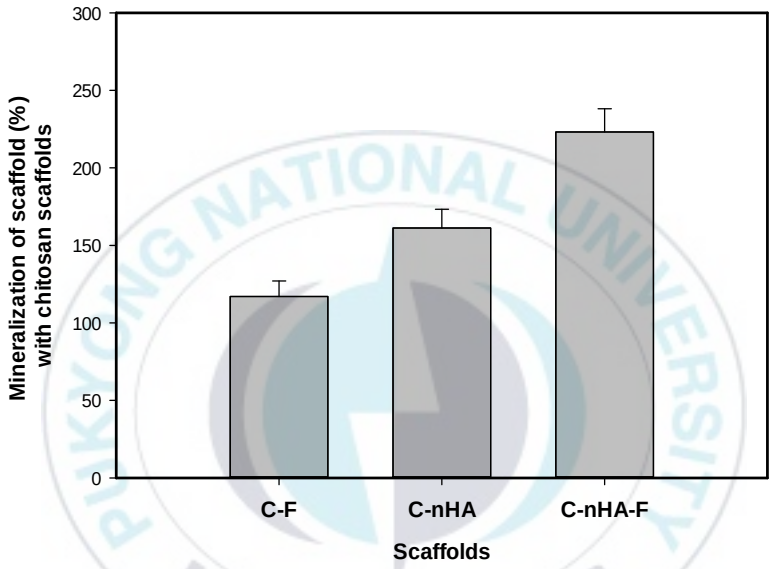

Figure 26. Shows the results of mineralization of C, C-F, C-nHA- C-nHA-F in 28th days.

3.6. Conclusion

Here we report a 3D structured functionalized scaffold made of chitosan-fucoidan and nHA isolated from salmon fish bone. FTIR and XRD analysis both confirm the presence of the carbonated groups in the isolated nHA and cytotoxity results confirm higher level of cell viability and increasing proliferation as well mineral formation. We confirmed the differentiation of the stem cells in to osteoblast progenitors. Additionally, the periosteum derived mesenchymal stem cells were observed growing on the scaffold matrices without an elucidated toxic effect. The increase in proliferation in the C-nHA-F scaffold is attributed to the presence of fucoidan and the nHA induced stem cell differentiation to producing bone minerals. Therefore, we suggest that C-nHA-F holds much promise for application in bone tissue engineering based therapies.

Chapter 4.

4.1. Cumulative Summary and Conclusion

The work presented in this thesis has been focused on the isolation of Nano-hydroxyapatite from Salmon fish bone and it's functionalization in to a suitable Nanocomposite made of Chitosan and Fucoidan as constituent materials for use in bone tissue regeneration.

- Alkaline hydrolysis has been employed in the isolation of Nano-hydroxyapatite and we draw a conclusion that the isolated Nano-hydroxyapatite is pure in reference to the Joint committee on Powder Diffraction Standards (JCPDS 090432).
- The isolated Nano-hydroxyapatite has the capacity of inducing mesenchymal stem cell differentiation when used as a target to produce osteoblast cells.
- Using a freeze-drying method, we fabricated a nano-composite made of Nano-hydroxyapatite isolated from Salmon bone, Chitosan and Fucoidan.
- The nano-composite was biocompatible with a suitable micro-architecture for cell growth.
- The nanocomposite also modelled in mesenchymal stem cell differentiation producing bone minerals.

With further *in vivo* investigations and clinical trial studies these biomaterials presented here would be add important contributions in regenerative medicine especially, in bone tissue regeneration based therapeutics.

5.0. References

Abdallah, B. M., Haack-Sørensen, M., Fink, T., & Kassem, M. (2006). Inhibition of osteoblast differentiation but not adipocyte differentiation of mesenchymal stem cells by sera obtained from aged females. *Bone, 39*(1), 181-188.

Akkouch, A., Zhang, Z., & Rouabhia, M. (2014). Engineering bone tissue using human dental pulp stem cells and an osteogenic collagen-hydroxyapatite-poly (l-lactide-co-ε-caprolactone) scaffold. *Journal of Biomaterials Applications, 28*(6), 922-936.

Aleksandar, D., Nenad, I., Mariana, S., Danica, J., Dragan, U., & Zlatko, R. (2013). Synthesis and Characterization of Hydroxyapatite/Fullerenol Nanocomposites. *J. Nanosci. Nanotechnol, 13*, 1-5.

Alison, M. R., Poulsom, R., Jeffery, R., Dhillon, A. P., Quaglia, A., Jacob, J., . . . Wright, N. A. (2000). Cell differentiation: hepatocytes from non-hepatic adult stem cells. *Nature, 406*, 257.

Allo, B. A., Costa, D. O., Dixon, S. J., Mequanint, K., & Rizkalla, A. S. (2012). Bioactive and biodegradable nanocomposites and hybrid biomaterials for bone regeneration. *Journal of functional biomaterials, 3*(2), 432-463.

Ambre, A. H., Katti, D. R., & Katti, K. S. (2015). Biomineralized hydroxyapatite nanoclay composite scaffolds with polycaprolactone for stem cell-based bone tissue engineering. *Journal of Biomedical Materials Research Part A, 103*(6), 2077-2101.

Amna, T., Hassan, M. S., Khil, M.-S., & Hwang, I. (2014). Biological interactions of muscle precursor C2C12 cells with biomimetic nano-hydroxyapatite/poly (lactide-co-glycolide) scaffoldings. *Ceramics International, 40*(9), 14305-14311.

Anghileri, E., Marconi, S., Pignatelli, A., Cifelli, P., Galié, M., Sbarbati, A., . . . Bonetti, B. (2008). Neuronal differentiation potential of human adipose-derived mesenchymal stem cells. *Stem cells and development, 17*(5), 909-916.

Arora, P., Sindhu, A., Dilbaghi, N., Chaudhury, A., Rajakumar, G., & Rahuman, A. A. (2012). Nano-regenerative medicine towards clinical outcome of stem cell and tissue engineering in humans. *Journal of Cellular and Molecular Medicine, 16*(9), 1991-2000.

Arun, K. R., Sivashanmugam, A., Deepthi, S., Iseki, S., Chennazhi, K., Nair, S., & Jayakumar, R. (2015). Injectable Chitin-Poly (ε-caprolactone)/Nanohydroxyapatite Composite Microgels Prepared by Simple Regeneration Technique for Bone Tissue Engineering. *ACS applied materials & interfaces, 7*(18), 9399-9409.

Awad, H. A., Wickham, M. Q., Leddy, H. A., Gimble, J. M., & Guilak, F. (2004). Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials, 25*(16), 3211-3222.

Bäckesjö, C.-M., Li, Y., Lindgren, U., & Haldosén, L.-A. (2009). Activation of Sirt1 decreases adipocyte formation during osteoblast differentiation of mesenchymal stem cells. *Cells Tissues Organs, 189*(1-4), 93-97.

Bühring, H. J., Treml, S., Cerabona, F., De Zwart, P., Kanz, L., & Sobiesiak, M. (2009). Phenotypic characterization of distinct human bone marrow–derived MSC subsets. *Annals of the New York Academy of Sciences, 1176*(1), 124-134.

Bagheri-Khoulenjani, S., Mirzadeh, H., Etrati-Khosroshahi, M., & Ali Shokrgozar, M. (2013). Particle size modeling and morphology study of chitosan/gelatin/nanohydroxyapatite nanocomposite microspheres for bone tissue engineering. *Journal of Biomedical Materials Research Part A, 101*(6), 1758-1767.

Bahrololoom, M., Javidi, M., Javadpour, S., & Ma, J. (2009). Characterisation

of natural hydroxyapatite extracted from bovine cortical bone ash. *J. Ceram. Process. Res, 10*, 129-138.

Barakat, N. A., Khil, M. S., Omran, A., Sheikh, F. A., & Kim, H. Y. (2009). Extraction of pure natural hydroxyapatite from the bovine bones bio waste by three different methods. *Journal of materials processing technology, 209*(7), 3408-3415.

Bartsch Jr, G., Yoo, J. J., De Coppi, P., Siddiqui, M. M., Schuch, G., Pohl, H. G., . . . Atala, A. (2005). Propagation, expansion, and multilineage differentiation of human somatic stem cells from dermal progenitors. *Stem cells and development, 14*(3), 337-348.

Battula, V. L., Bareiss, P. M., Treml, S., Conrad, S., Albert, I., Hojak, S., . . . Skutella, T. (2007). Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation. *Differentiation, 75*(4), 279-291.

Bauer, T. W., & Muschler, G. F. (2000). Bone Graft Materials: An Overview of the Basic Science. *Clinical Orthopaedics and Related Research, 371*, 10-27.

Behnia, H., Khojasteh, A., Kiani, M. T., Khoshzaban, A., Abbas, F. M., Bashtar, M., & Dashti, S. G. (2013). Bone regeneration with a combination of nanocrystalline hydroxyapatite silica gel, platelet-rich growth factor, and mesenchymal stem cells: a histologic study in rabbit calvaria. *Oral surgery, oral medicine, oral pathology and oral radiology, 115*(2), e7-e15.

Behravesh, E., & Mikos, A. G. (2003). Three-dimensional culture of differentiating marrow stromal osteoblasts in biomimetic poly (propylene fumarate-co-ethylene glycol)-based macroporous hydrogels. *Journal of Biomedical Materials Research Part A, 66*(3), 698-706.

Bernardo, M. E., Zaffaroni, N., Novara, F., Cometa, A. M., Avanzini, M. A., Moretta, A., . . . Daidone, M. G. (2007). Human bone marrow–derived

mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Research, 67*(19), 9142-9149.

Bianco, P., & Robey, P. G. (2001). Stem cells in tissue engineering. *Nature, 414*(6859), 118-121.

Boos, A. M., Weigand, A., Deschler, G., Gerber, T., Arkudas, A., Kneser, U., . . . Beier, J. P. (2014). Autologous serum improves bone formation in a primary stable silica-embedded nanohydroxyapatite bone substitute in combination with mesenchymal stem cells and rhBMP-2 in the sheep model. *International journal of nanomedicine, 9*, 5317.

Bose, S., Roy, M., & Bandyopadhyay, A. (2012). Recent advances in bone tissue engineering scaffolds. *Trends in Biotechnology, 30*(10), 546-554.

Bosnakovski, D., Mizuno, M., Kim, G., Takagi, S., Okumura, M., & Fujinaga, T. (2006). Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. *Biotechnology and Bioengineering, 93*, 1152-1163.

Boulila, S., Elfeki, A., Oudadesse, H., & Elfeki, H. (2015). Substitution effects of a carbonated hydroxyapatite biomaterial against intoxication chloride nickel-exposed rats. *Toxicology Mechanisms and Methods*(0), 1-11.

Boutinguiza, M., Pou, J., Comesaña, R., Lusquiños, F., De Carlos, A., & León, B. (2012). Biological hydroxyapatite obtained from fish bones. *Materials Science and Engineering: C, 32*(3), 478-486.

Brighton, C. T., Lorich, D. G., Kupcha, R., Reilly, T. M., Jones, A. R., & ROBERT A WOODBURY, I. (1992). The pericyte as a possible osteoblast progenitor cell. *Clinical Orthopaedics and Related Research, 275*, 287-299.

Burg, K. J., Porter, S., & Kellam, J. F. (2000). Biomaterial developments for bone tissue engineering. *Biomaterials, 21*(23), 2347-2359.

Buschmann, J., Härter, L., Gao, S., Hemmi, S., Welti, M., Hild, N., . . . Werner,

C. M. (2012). Tissue engineered bone grafts based on biomimetic nanocomposite PLGA/amorphous calcium phosphate scaffold and human adipose-derived stem cells. *Injury, 43*(10), 1689-1697.

Caballero, M., Reed, C. R., Madan, G., & van Aalst, J. A. (2010). Osteoinduction in umbilical cord-and palate periosteum-derived mesenchymal stem cells. *Annals of Plastic Surgery, 64*(5), 605-609.

Cai, X., Tong, H., Shen, X., Chen, W., Yan, J., & Hu, J. (2009). Preparation and characterization of homogeneous chitosan–polylactic acid/hydroxyapatite nanocomposite for bone tissue engineering and evaluation of its mechanical properties. *Acta biomaterialia, 5*(7), 2693-2703.

Calvi, L., Adams, G., Weibrecht, K., Weber, J., Olson, D., Knight, M., . . . Bringhurst, F. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature, 425*(6960), 841-846.

Cancedda, R., Dozin, B., Giannoni, P., & Quarto, R. (2003). Tissue engineering and cell therapy of cartilage and bone. *Matrix Biology, 22*(1), 81-91.

Cao, L.-y., Zhang, C.-b., & Huang, J.-f. (2005). Synthesis of hydroxyapatite nanoparticles in ultrasonic precipitation. *Ceramics International, 31*(8), 1041-1044.

Carvalho, J. L., de Goes, A. M., Gomes, D. A., & de Carvalho, P. H. (2013). *Innovative Strategies for Tissue Engineering, Advances in Biomaterials Science and Biomedical Applications, Prof. Rosario Pignatello (Ed.), ISBN: 978-953-51-1051-4, InTech, DOI: 10.5772/53337. Available from: http://www.intechopen.com/books/advances-in-biomaterials-science-and-biomedical-applications/innovative-strategies-for-tissue-engineering*: INTECH Open Access Publisher.

Chang, N.-J., Lin, C.-C., Li, C.-F., Wang, D.-A., Issariyaku, N., & Yeh, M.-L. (2012). The combined effects of continuous passive motion treatment and acellular PLGA implants on osteochondral regeneration in the rabbit. *Biomaterials, 33*(11), 3153-3163.

Chang, Y. L., Stanford, C. M., & Keller, J. C. (2000). Calcium and phosphate supplementation promotes bone cell mineralization: Implications for hydroxyapatite (HA)-enhanced bone formation. *Journal of Biomedical Materials Research, 52*(2), 270-278.

Chattanathan, S., Clement, T., Kanel, S., Barnett, M., & Chatakondi, N. (2013). Remediation of uranium-contaminated groundwater by sorption onto hydroxyapatite derived from catfish bones. *Water, Air, and Soil Pollution, 224*(2), 1-9.

Chaubey, A., Ross, K. J., Leadbetter, R. M., & Burg, K. J. (2008). Surface patterning: tool to modulate stem cell differentiation in an adipose system. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 84*(1), 70-78.

Chen, M., Patra, P. K., Warner, S. B., & Bhowmick, S. (2007). Role of fiber diameter in adhesion and proliferation of NIH 3T3 fibroblast on electrospun polycaprolactone scaffolds. *Tissue Engineering, 13*(3), 579-587.

Chevallier, N., Anagnostou, F., Zilber, S., Bodivit, G., Maurin, S., Barrault, A., . . . Rouard, H. (2010). Osteoblastic differentiation of human mesenchymal stem cells with platelet lysate. *Biomaterials, 31*(2), 270-278.

Cho, Y.-S., Jung, W.-K., Kim, J.-A., Choi, I.-W., & Kim, S.-K. (2009). Beneficial effects of fucoidan on osteoblastic MG-63 cell differentiation. *Food Chemistry, 116*(4), 990-994.

Chua, K.-N., Chai, C., Lee, P.-C., Tang, Y.-N., Ramakrishna, S., Leong, K. W., & Mao, H.-Q. (2006). Surface-aminated electrospun nanofibers enhance adhesion and expansion of human umbilical cord blood hematopoietic stem/progenitor cells. *Biomaterials, 27*(36), 6043-6051.

Coelho, J., Moreira, J. A., Almeida, A., & Monteiro, F. (2010). Synthesis and characterization of HAp nanorods from a cationic surfactant template method. *Journal of Materials Science: Materials in Medicine, 21*(9),

2543-2549.

Coelho, T., Nogueira, E., Weinand, W., Lima, W., Steimacher, A., Medina, A., . . . Bento, A. (2007). Thermal properties of natural nanostructured hydroxyapatite extracted from fish bone waste. *Journal of applied physics, 101*(8), 084701.

D'Ippolito, G., Schiller, P. C., Ricordi, C., Roos, B. A., & Howard, G. A. (1999). Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. *Journal of Bone and Mineral Research, 14*(7), 1115-1122.

De Bari, C., Dell'Accio, F., & Luyten, F. P. (2001). Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. *Arthritis and Rheumatism, 44*(1), 85-95.

De Bari, C., Dell'Accio, F., Tylzanowski, P., & Luyten, F. P. (2001). Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis and Rheumatism, 44*(8), 1928-1942.

De Bari, C., Dell'Accio, F., Vandenabeele, F., Vermeesch, J. R., Raymackers, J.-M., & Luyten, F. P. (2003). Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *The Journal of cell biology, 160*(6), 909-918.

Debnath, T., Ghosh, S., Potlapuvu, U. S., Kona, L., Kamaraju, S. R., Sarkar, S., . . . Chelluri, L. K. (2015). Proliferation and Differentiation Potential of Human Adipose-Derived Stem Cells Grown on Chitosan Hydrogel. *PloS One, 10*(3).

Di Liddo, R., Paganin, P., Lora, S., Dalzoppo, D., Giraudo, C., Miotto, D., . . . Bianchi, E. (2014). Poly-ε-caprolactone composite scaffolds for bone repair. *International Journal of Molecular Medicine, 34*(6), 1537-1546.

Di Martino, A., Sittinger, M., & Risbud, M. V. (2005). Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials, 26*(30), 5983-5990.

Diefenderfer, D. L., & Brighton, C. T. (2000). Microvascular pericytes express aggrecan message which is regulated by BMP-2. *Biochemical and Biophysical Research Communications, 269*(1), 172-178.

Djouad, F., Bony, C., Haupl, T., Uzé, G., Lahlou, N., Louis-Plence, P., . . . Sany, J. (2005). Transcriptional profiles discriminate bone marrow-derived and synovium-derived mesenchymal stem cells. *Arthritis Research & Therapy, 7*(6), R1304-R1315.

Drosse, I., Volkmer, E., Capanna, R., De Biase, P., Mutschler, W., & Schieker, M. (2008). Tissue engineering for bone defect healing: an update on a multi-component approach. *Injury, 39*, S9-S20.

Dvir, T., Timko, B. P., Kohane, D. S., & Langer, R. (2011). Nanotechnological strategies for engineering complex tissues. *Nature nanotechnology, 6*(1), 13-22.

El Tamer, M., & Reis, R. (2009). Progenitor and stem cells for bone and cartilage regeneration. *Journal of Tissue Engineering and Regenerative Medicine, 3*(5), 327-337.

Emerich, D. F., & Thanos, C. G. (2003). Nanotechnology and medicine. *Expert Opinion on Biological Therapy, 3*(4), 655-663.

Eshraghi, S., & Das, S. (2012). Micromechanical finite-element modeling and experimental characterization of the compressive mechanical properties of polycaprolactone–hydroxyapatite composite scaffolds prepared by selective laser sintering for bone tissue engineering. *Acta Biomaterialia, 8*(8), 3138-3143.

Eyckmans, J., & Luyten, F. P. (2006). Species specificity of ectopic bone formation using periosteum-derived mesenchymal progenitor cells. *Tissue Engineering, 12*(8), 2203-2213.

Faßbender, M., Minkwitz, S., Strobel, C., Schmidmaier, G., & Wildemann, B. (2014). Stimulation of bone healing by sustained bone morphogenetic protein 2 (BMP-2) delivery. *International journal of molecular sciences, 15*(5), 8539-8552.

Feng, P., Niu, M., Gao, C., Peng, S., & Shuai, C. (2014). A novel two-step sintering for nano-hydroxyapatite scaffolds for bone tissue engineering. *Scientific Reports, 4*.

Ferretti, C., Borsari, V., Falconi, M., Gigante, A., Lazzarini, R., Fini, M., . . . Mattioli-Belmonte, M. (2012). Human periosteum-derived stem cells for tissue engineering applications: the role of VEGF. *Stem Cell Reviews and Reports, 8*(3), 882-890.

Flynn, A., Barry, F., & O'brien, T. (2007). UC blood-derived mesenchymal stromal cells: an overview. *Cytotherapy, 9*(8), 717-726.

Fricain, J. C., Schlaubitz, S., Le Visage, C., Arnault, I., Derkaoui, S. M., Siadous, R., . . . Renard, M. (2013). A nano-hydroxyapatite–pullulan/dextran polysaccharide composite macroporous material for bone tissue engineering. *Biomaterials, 34*(12), 2947-2959.

Frohbergh, M. E., Katsman, A., Botta, G. P., Lazarovici, P., Schauer, C. L., Wegst, U. G., & Lelkes, P. I. (2012). Electrospun hydroxyapatite-containing chitosan nanofibers crosslinked with genipin for bone tissue engineering. *Biomaterials, 33*(36), 9167-9178.

Gómez-Guillén, M., Giménez, B., López-Caballero, M. a., & Montero, M. (2011). Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids, 25*(8), 1813-1827.

Gang, E., Jeong, J., Han, S., Yan, Q., Jeon, C., & Kim, H. (2006). In vitro endothelial potential of human UC blood-derived mesenchymal stem cells. *Cytotherapy, 8*(3), 215-227.

Gao, P., Zhang, H., Xiao, X., Liu, Y., Geng, L., Yuan, Y., . . . Lu, J. (2015). An Innovative Osteo-Regenerator Based on Beta-Tricalcium Phosphate Granules for Bone Tissue Engineering. *Journal of Biomaterials and Tissue Engineering, 5*(1), 50-55.

GĂśtz, W., Lenz, S., Reichert, C., Henkel, K., Pernicka, L., Gundlach, K., . . . Heinemann, F. (2011). A preliminary study in osteoinduction by a nano-crystalline hydroxyapatite in the mini pig. *Folia Histochemica et*

Cytobiologica, 48(4), 589-588.

Ge, S., Zhao, N., Wang, L., Liu, H., & Yang, P. (2013). Effects of hydroxyapatite nanostructure on channel surface of porcine acellular dermal matrix scaffold on cell viability and osteogenic differentiation of human periodontal ligament stem cells. *International journal of nanomedicine, 8*, 1887.

Ge, S., Zhao, N., Wang, L., Yu, M., Liu, H., Song, A., . . . Yang, P. (2012). Bone repair by periodontal ligament stem cellseeded nanohydroxyapatite-chitosan scaffold. *International journal of nanomedicine, 7*, 5405.

Gelinsky, M., Welzel, P., Simon, P., Bernhardt, A., & König, U. (2008). Porous three-dimensional scaffolds made of mineralised collagen: preparation and properties of a biomimetic nanocomposite material for tissue engineering of bone. *Chemical Engineering Journal, 137*(1), 84-96.

Gimble, J., & Guilak, F. (2003). Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy, 5*(5), 362-369.

Goldberg, M., Langer, R., & Jia, X. (2007). Nanostructured materials for applications in drug delivery and tissue engineering. *Journal of Biomaterials Science, Polymer Edition, 18*(3), 241-268.

Gomes, S., Leonor, I. B., Mano, J. F., Reis, R. L., & Kaplan, D. L. (2012). Natural and genetically engineered proteins for tissue engineering. *Progress in polymer science, 37*(1), 1-17.

Griffith, L. G. (2002). Emerging design principles in biomaterials and scaffolds for tissue engineering. *Annals of the New York Academy of Sciences, 961*(1), 83-95.

Griffith, L. G., & Naughton, G. (2002). Tissue engineering--current challenges and expanding opportunities. *Science, 295*(5557), 1009-1014.

Grisendi, G., Bussolari, R., Cafarelli, L., Petak, I., Rasini, V., Veronesi, E., . . . Barti-Juhasz, H. (2010). Adipose-derived mesenchymal stem cells as

stable source of tumor necrosis factor–related apoptosis-inducing ligand delivery for cancer therapy. *Cancer Research, 70*(9), 3718-3729.

Grzesiak, J., Kolankowski, J., & Marycz, K. (2015). Osteogenic Differentiation of Canine Adipose Stem Cells Cultured in Alginate-Fibrin-Based Hydrogel. *Journal of Biomaterials and Tissue Engineering, 5*(9), 703-710.

Gu, Y., Huang, W., Rahaman, M. N., & Day, D. E. (2013). Bone regeneration in rat calvarial defects implanted with fibrous scaffolds composed of a mixture of silicate and borate bioactive glasses. *Acta Biomaterialia, 9*(11), 9126-9136.

Guo, G., Sun, Y., Wang, Z., & Guo, H. (2005). Preparation of hydroxyapatite nanoparticles by reverse microemulsion. *Ceramics International, 31*(6), 869-872.

Guo, J., Wang, Y., Cao, C., Dziak, R., Preston, B., & Guan, G. (2013). Human periodontal ligament cells reaction on a novel hydroxyapatite–collagen scaffold. *Dental Traumatology, 29*(2), 103-109.

Häggström, M. (2014). Medical gallery of Mikael Hggstrm 2014. *Wikiversity J Med, 1*(2).

Haberko, K., Bućko, M. M., Brzezińska-Miecznik, J., Haberko, M., Mozgawa, W., Panz, T., . . . Zarębski, J. (2006). Natural hydroxyapatite—its behaviour during heat treatment. *Journal of the European Ceramic Society, 26*(4), 537-542.

Hegewald, A., Ringe, J., Bartel, J., Krüger, I., Notter, M., Barnewitz, D., . . . Sittinger, M. (2004). Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study. *Tissue and Cell, 36*(6), 431-438.

Hickey, D. J., Ercan, B., Sun, L., & Webster, T. J. (2015). Adding MgO nanoparticles to hydroxyapatite–PLLA nanocomposites for improved bone tissue engineering applications. *Acta Biomaterialia, 14*, 175-184.

Hild, N., Schneider, O. D., Mohn, D., Luechinger, N. A., Koehler, F. M.,

Hofmann, S., . . . Stark, W. J. (2011). Two-layer membranes of calcium phosphate/collagen/PLGA nanofibres: in vitro biomineralisation and osteogenic differentiation of human mesenchymal stem cells. *Nanoscale, 3*(2), 401-409.

Hile, D. D., Amirpour, M. L., Akgerman, A., & Pishko, M. V. (2000). Active growth factor delivery from poly (d, l-lactide-co-glycolide) foams prepared in supercritical CO 2. *Journal of Controlled Release, 66*(2), 177-185.

Hollister, S. J. (2005). Porous scaffold design for tissue engineering. *Nature materials, 4*(7), 518-524.

Hoogduijn, M., Crop, M., Peeters, A., Van Osch, G., Balk, A., Ijzermans, J., . . . Baan, C. (2007). Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. *Stem cells and development, 16*(4), 597-604.

Horch, R. E., Boos, A. M., Quan, Y., Bleiziffer, O., Detsch, R., Boccaccini, A. R., . . . Arkudas, A. (2013). Cancer research by means of tissue engineering–is there a rationale? *Journal of Cellular and Molecular Medicine, 17*(10), 1197-1206.

Horch, R. E., Kneser, U., Polykandriotis, E., Schmidt, V. J., Sun, J., & Arkudas, A. (2012). Tissue engineering and regenerative medicine–where do we stand? *Journal of Cellular and Molecular Medicine, 16*(6), 1157-1165.

Horwitz, E. M., Gordon, P. L., Koo, W. K., Marx, J. C., Neel, M. D., McNall, R. Y., . . . Hofmann, T. (2002). Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proceedings of the National Academy of Sciences, 99*(13), 8932-8937.

Horwitz, E. M., Prockop, D. J., Fitzpatrick, L. A., Koo, W. W., Gordon, P. L., Neel, M., . . . Pyeritz, R. E. (1999). Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nature Medicine, 5*(3), 309-313.

Hoyer, B., Bernhardt, A., Heinemann, S., Stachel, I., Meyer, M., & Gelinsky, M. (2012). Biomimetically mineralized salmon collagen scaffolds for application in bone tissue engineering. *Biomacromolecules, 13*(4), 1059-1066.

Hsiao, S. T.-F., Asgari, A., Lokmic, Z., Sinclair, R., Dusting, G. J., Lim, S. Y., & Dilley, R. J. (2011). Comparative analysis of paracrine factor expression in human adult mesenchymal stem cells derived from bone marrow, adipose, and dermal tissue. *Stem cells and development, 21*(12), 2189-2203.

Hu, Q., Li, B., Wang, M., & Shen, J. (2004). Preparation and characterization of biodegradable chitosan/hydroxyapatite nanocomposite rods via in situ hybridization: a potential material as internal fixation of bone fracture. *Biomaterials, 25*(5), 779-785.

Hu, S., Zeng, Y., Yang, S., Qin, H., Cai, H., & Wang, J. (2015). Application of Graphene Based Nanotechnology in Stem Cells Research. *Journal of Nanoscience and Nanotechnology, 15*(9), 6327-6341.

Hu, Y., Hollinger, J., & Marra, K. (2001). Controlled release from coated polymer microparticles embedded in tissue-engineered scaffolds. *Journal of Drug Targeting, 9*(6), 431-438.

Huang, X., Bai, S., Lu, Q., Liu, X., Liu, S., & Zhu, H. (2014). Osteoinductive-nanoscaled silk/HA composite scaffolds for bone tissue engineering application. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*.

Huang, Y.-C., Hsiao, P.-C., & Chai, H.-J. (2011). Hydroxyapatite extracted from fish scale: Effects on MG63 osteoblast-like cells. *Ceramics International, 37*(6), 1825-1831.

Huang, Y.-C., & Li, R.-Y. (2014). Preparation and characterization of antioxidant nanoparticles composed of chitosan and fucoidan for antibiotics delivery. *Marine Drugs, 12*(8), 4379-4398.

Huang, Z., Yu, B., Feng, Q., Li, S., Chen, Y., & Luo, L. (2011). In situ-forming

chitosan/nano-hydroxyapatite/collagen gel for the delivery of bone marrow mesenchymal stem cells. *Carbohydrate polymers, 85*(1), 261-267.

Hubbell, J. A. (1995). Biomaterials in tissue engineering. *Nature Biotechnology, 13*(6), 565-576.

Huh, J.-B., Yang, J.-J., Choi, K.-H., Bae, J. H., Lee, J.-Y., Kim, S.-E., & Shin, S.-W. (2015). Effect of rhBMP-2 Immobilized Anorganic Bovine Bone Matrix on Bone Regeneration. *International journal of molecular sciences, 16*(7), 16034-16052.

Hui, J. H., Li, L., Teo, Y.-H., Ouyang, H.-W., & Lee, E.-H. (2005). Comparative study of the ability of mesenchymal stem cells derived from bone marrow, periosteum, and adipose tissue in treatment of partial growth arrest in rabbit. *Tissue Engineering, 11*(5-6), 904-912.

Huri, P. Y. (2015). Effect of Culture Conditions on the Multinucleation of Human Adipose-Derived Stem Cells. *Journal of Biomaterials and Tissue Engineering, 5*(3), 234-240.

Hwang, Y.-S., Cho, J., Tay, F., Heng, J. Y., Ho, R., Kazarian, S. G., . . . Mantalaris, A. (2009). The use of murine embryonic stem cells, alginate encapsulation, and rotary microgravity bioreactor in bone tissue engineering. *Biomaterials, 30*(4), 499-507.

Ibara, A., Miyaji, H., Fugetsu, B., Nishida, E., Takita, H., Tanaka, S., . . . Kawanami, M. (2013). Osteoconductivity and biodegradability of collagen scaffold coated with nano-β-TCP and fibroblast growth factor 2. *Journal of Nanomaterials, 2013*, 46.

Ito, T., Suzuki, A., Okabe, M., Imai, E., & Hori, M. (2001). Application of bone marrow-derived stem cells in experimental nephrology. *Nephron Experimental Nephrology, 9*(6), 444-450.

Jackson, D. W., & Simon, T. M. (1999). Tissue engineering principles in orthopaedic surgery. *Clinical Orthopaedics and Related Research, 367*, S31-S45.

Jang, H.-O., Park, Y.-S., Lee, J.-H., Seo, J.-B., Koo, K.-I., Jeong, S.-C., . . . Yun, I. (2007). Effect of extracts from safflower seeds on osteoblast differentiation and intracellular calcium ion concentration in MC3T3-E1 cells. *Natural product research, 21*(9), 787-797.

Janus, A. M., Faryna, M., Haberko, K., Rakowska, A., & Panz, T. (2008). Chemical and microstructural characterization of natural hydroxyapatite derived from pig bones. *Microchimica Acta, 161*(3-4), 349-353.

Jarudilokkul, S., Tanthapanichakoon, W., & Boonamnuayvittaya, V. (2007). Synthesis of hydroxyapatite nanoparticles using an emulsion liquid membrane system. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 296*(1), 149-153.

Jeong, H.-S., Venkatesan, J., & Kim, S.-K. (2013). Hydroxyapatite-fucoidan nanocomposites for bone tissue engineering. *International Journal of Biological Macromolecules, 57*, 138-141.

Jin, L., Lee, J. H., Jin, O. S., Shin, Y. C., Kim, M. J., Hong, S. W., . . . Han, D.-W. (2015). Stimulated Osteogenic Differentiation of Human Mesenchymal Stem Cells by Reduced Graphene Oxide. *Journal of Nanoscience and Nanotechnology, 15*(10), 7966-7970.

Jin, L., Wan, Y., Shimer, A. L., Shen, F. H., & Li, X. J. (2012). Intervertebral disk-like biphasic scaffold—demineralized bone matrix cylinder and poly (polycaprolactone triol malate)—for interbody spine fusion. *Journal of tissue engineering*, 2041731412454420.

Joannides, A., Gaughwin, P., Schwiening, C., Majed, H., Sterling, J., Compston, A., & Chandran, S. (2004). Efficient generation of neural precursors from adult human skin: astrocytes promote neurogenesis from skin-derived stem cells. *The Lancet, 364*(9429), 172-178.

Jones, E. A., Crawford, A., English, A., Henshaw, K., Mundy, J., Corscadden, D., . . . McGonagle, D. (2008). Synovial fluid mesenchymal stem cells in health and early osteoarthritis: Detection and functional evaluation at the single-cell level. *Arthritis and Rheumatism, 58*(6), 1731-1740.

Joschek, S., Nies, B., Krotz, R., & Göpferich, A. (2000). Chemical and physicochemical characterization of porous hydroxyapatite ceramics made of natural bone. *Biomaterials, 21*(16), 1645-1658.

Kanda, Y., Hinata, T., Kang, S. W., & Watanabe, Y. (2011). Reactive oxygen species mediate adipocyte differentiation in mesenchymal stem cells. *Life Sciences, 89*(7), 250-258.

Kang, K., Kim, S., Oh, Y., Yu, J., Kim, K., Park, H., . . . Han, H. (2005). A 37-year-old spinal cord-injured female patient, transplanted of multipotent stem cells from human UC blood, with improved sensory perception and mobility, both functionally and morphologically: a case study. *Cytotherapy, 7*(4), 368-373.

Karageorgiou, V., & Kaplan, D. (2005). Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials, 26*(27), 5474-5491.

Khademhosseini, A., Langer, R., Borenstein, J., & Vacanti, J. P. (2006). Microscale technologies for tissue engineering and biology. *Proceedings of the National Academy of Sciences of the United States of America, 103*(8), 2480-2487.

Kim, B.-S., Kang, H. J., Yang, S.-S., & Lee, J. (2014). Comparison of in vitro and in vivo bioactivity: cuttlefish-bone-derived hydroxyapatite and synthetic hydroxyapatite granules as a bone graft substitute. *Biomedical Materials, 9*(2), 025004.

Kim, B. S., Kang, H.-J., Park, J.-Y., & Lee, J. (2015). Fucoidan promotes osteoblast differentiation via JNK-and ERK-dependent BMP2–Smad 1/5/8 signaling in human mesenchymal stem cells. *Experimental and Molecular Medicine, 47*(1), e128.

Kim, B. S., Kim, J. S., Chung, Y. S., Sin, Y. W., Ryu, K. H., Lee, J., & You, H. K. (2013). Growth and osteogenic differentiation of alveolar human bone marrow-derived mesenchymal stem cells on chitosan/hydroxyapatite composite fabric. *Journal of Biomedical Materials Research Part A, 101*(6), 1550-1558.

Kim, D.-H., Shin, K.-K., Jung, J. S., Chun, H. H., Park, S. S., Lee, J. K., . . . Yoon, S.-Y. (2015). The Role of Magnesium Ion Substituted Biphasic Calcium Phosphate Spherical Micro-Scaffolds in Osteogenic Differentiation of Human Adipose Tissue-Derived Mesenchymal Stem Cells. *Journal of Nanoscience and Nanotechnology, 15*(8), 5520-5523.

Kim, H., Lee, A., Jung, W.-K., & Jeon, T. J. (2015). Effects of fucoidan on cell morphology and migration in osteoblasts. *Food Science and Biotechnology, 24*(2), 699-704.

Kim, H. J., Kim, U.-J., Vunjak-Novakovic, G., Min, B.-H., & Kaplan, D. L. (2005). Influence of macroporous protein scaffolds on bone tissue engineering from bone marrow stem cells. *Biomaterials, 26*(21), 4442-4452.

Kim, M. Y., Im, S.-W., & Park, H. M. (2015). The demographic changes of menopausal and geripausal women in Korea. *Journal of bone metabolism, 22*(1), 23-28.

Kim, S.-K., & Cho, Y.-S. (2009). Pharmaceutical compositions containing fucoidan for stimulating and activating osteogenesis: Google Patents.

Kim, S. M., Lim, J. Y., Park, S. I., Jeong, C. H., Oh, J. H., Jeong, M., . . . Jeun, S.-S. (2008). Gene therapy using TRAIL-secreting human umbilical cord blood–derived mesenchymal stem cells against intracranial glioma. *Cancer Research, 68*(23), 9614-9623.

Kim, W.-S., Park, B.-S., Park, S.-H., Kim, H.-K., & Sung, J.-H. (2009). Antiwrinkle effect of adipose-derived stem cell: activation of dermal fibroblast by secretory factors. *Journal of Dermatological Science, 53*(2), 96-102.

Kingsley, J. D., Ranjan, S., Dasgupta, N., & Saha, P. (2013). Nanotechnology for tissue engineering: Need, techniques and applications. *Journal of Pharmacy Research, 7*(2), 200-204.

Koç, A., Finkenzeller, G., Elçin, A. E., Stark, G. B., & Elçin, Y. M. (2014). Evaluation of adenoviral vascular endothelial growth factor-activated

chitosan/hydroxyapatite scaffold for engineering vascularized bone tissue using human osteoblasts: In vitro and in vivo studies. *Journal of Biomaterials Applications, 29*(5), 748-760.

Ko, Y.-M., Myung, S.-W., & Kim, B.-H. (2015). O2/Ar Plasma Treatment for Enhancing the Biocompatibility of Hydroxyapatite Nanopowder and Polycaprolactone Composite Film. *Journal of Nanoscience and Nanotechnology, 15*(8), 6048-6052.

Kongsri, S., Janpradit, K., Buapa, K., Techawongstien, S., & Chanthai, S. (2013). Nanocrystalline hydroxyapatite from fish scale waste: Preparation, characterization and application for selenium adsorption in aqueous solution. *Chemical Engineering Journal, 215*, 522-532.

Kopen, G. C., Prockop, D. J., & Phinney, D. G. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proceedings of the National Academy of Sciences, 96*(19), 10711-10716.

Kulterer, B., Friedl, G., Jandrositz, A., Sanchez-Cabo, F., Prokesch, A., Paar, C., . . . Trajanoski, Z. (2007). Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics, 8*(1), 70.

Kuznetsov, S. A., Krebsbach, P. H., Satomura, K., Kerr, J., Riminucci, M., Benayahu, D., & Robey, P. G. (1997). Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. *Journal of Bone and Mineral Research, 12*(9), 1335-1347.

Lü, X. Y., Fan, Y. B., Gu, D., & Cui, W. (2007). *Preparation and characterization of natural hydroxyapatite from animal hard tissues.* Paper presented at the Key Engineering Materials.

Landi, E., Celotti, G., Logroscino, G., & Tampieri, A. (2003). Carbonated hydroxyapatite as bone substitute. *Journal of the European Ceramic Society, 23*(15), 2931-2937.

Lao, L., Wang, Y., Zhu, Y., Zhang, Y., & Gao, C. (2011). Poly (lactide-co-

glycolide)/hydroxyapatite nanofibrous scaffolds fabricated by electrospinning for bone tissue engineering. *Journal of Materials Science: Materials in Medicine, 22*(8), 1873-1884.

Laschke, M. W., Harder, Y., Amon, M., Martin, I., Farhadi, J., Ring, A., . . . Junker, D. (2006). Angiogenesis in tissue engineering: breathing life into constructed tissue substitutes. *Tissue Engineering, 12*(8), 2093-2104.

Lee, C. H., Shah, B., Moioli, E. K., & Mao, J. J. (2010). CTGF directs fibroblast differentiation from human mesenchymal stem/stromal cells and defines connective tissue healing in a rodent injury model. *The Journal of clinical investigation, 120*(9), 3340.

LeGeros, R. Z. (2002). Properties of osteoconductive biomaterials: calcium phosphates. *Clinical Orthopaedics and Related Research, 395*, 81-98.

Li, J., Li, Y., Ma, S., Gao, Y., Zuo, Y., & Hu, J. (2010). Enhancement of bone formation by BMP-7 transduced MSCs on biomimetic nano-hydroxyapatite/polyamide composite scaffolds in repair of mandibular defects. *Journal of Biomedical Materials Research Part A, 95*(4), 973-981.

Li, Z., Yubao, L., Aiping, Y., Xuelin, P., Xuejiang, W., & Xiang, Z. (2005). Preparation and in vitro investigation of chitosan/nano-hydroxyapatite composite used as bone substitute materials. *Journal of Materials Science: Materials in Medicine, 16*(3), 213-219.

Liao, S., Cui, F., Zhang, W., & Feng, Q. (2004). Hierarchically biomimetic bone scaffold materials: nano-HA/collagen/PLA composite. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 69*(2), 158-165.

Lima, W., Weinand, W., dos Santos, O., Paesano, A., & Ortega, F. (2005). *Effect of the calcination time of fish bones in the synthesis of hydroxyapatite.* Paper presented at the Materials science forum.

Lin, K., Chen, L., & Chang, J. (2012). Fabrication of Dense Hydroxyapatite

Nanobioceramics with Enhanced Mechanical Properties via Two-Step Sintering Process. *International Journal of Applied Ceramic Technology, 9*(3), 479-485.

Liu, F., Meng, Q., Chen, C., Cui, F., Liu, Y., & Lee, I.-S. (2015). Loading Collagen and Hydroxycholesterol Within Biomimetic Mineral Onto Titanium: Synergistically Promoting Osteogenic Expression of Mesenchymal Stem Cells. *Journal of Biomaterials and Tissue Engineering, 5*(2), 169-176.

Liu, H., Cheng, J., Chen, F., Hou, F., Bai, D., Xi, P., & Zeng, Z. (2014). Biomimetic and cell-mediated mineralization of hydroxyapatite by carrageenan functionalized graphene oxide. *ACS applied materials & interfaces, 6*(5), 3132-3140.

Liu, H., Mao, J., Yao, K., Yang, G., Cui, L., & Cao, Y. (2004). A study on a chitosan-gelatin-hyaluronic acid scaffold as artificial skin in vitro and its tissue engineering applications. *Journal of Biomaterials Science, Polymer Edition, 15*(1), 25-40.

Liu, H., Xu, G. W., Wang, Y. F., Zhao, H. S., Xiong, S., Wu, Y., . . . Xie, D. H. (2015). Composite scaffolds of nano-hydroxyapatite and silk fibroin enhance mesenchymal stem cell-based bone regeneration via the interleukin 1 alpha autocrine/paracrine signaling loop. *Biomaterials, 49*, 103-112.

Liu, Y., Lim, J., & Teoh, S.-H. (2013). Review: development of clinically relevant scaffolds for vascularised bone tissue engineering. *Biotechnology advances, 31*(5), 688-705.

Loher, S., Reboul, V., Brunner, T. J., Simonet, M., Dora, C., Neuenschwander, P., & Stark, W. J. (2006). Improved degradation and bioactivity of amorphous aerosol derived tricalcium phosphate nanoparticles in poly (lactide-co-glycolide). *Nanotechnology, 17*(8), 2054.

Lou, T., Leung, M., Wang, X., Chang, J. Y. F., Tsao, C. T., Sham, J. G. C., . . . Zhang, M. (2014). Bi-layer scaffold of chitosan/PCL-nanofibrous mat

and PLLA-microporous disc for skin tissue engineering. *Journal of Biomedical Nanotechnology, 10*(6), 1105-1113.

Müller, P., Bulnheim, U., Diener, A., Lüthen, F., Teller, M., Klinkenberg, E. D., . . . Steinhoff, G. (2008). Calcium phosphate surfaces promote osteogenic differentiation of mesenchymal stem cells. *Journal of Cellular and Molecular Medicine, 12*(1), 281-291.

Ma, K., Chan, C. K., Liao, S., Hwang, W. Y., Feng, Q., & Ramakrishna, S. (2008). Electrospun nanofiber scaffolds for rapid and rich capture of bone marrow-derived hematopoietic stem cells. *Biomaterials, 29*(13), 2096-2103.

Madihally, S. V., & Matthew, H. W. (1999). Porous chitosan scaffolds for tissue engineering. *Biomaterials, 20*(12), 1133-1142.

Malina, D., Biernat, K., & Sobczak-Kupiec, A. (2012). Studies on sintering process of synthetic hydroxyapatite. *Acta. Biochim. Pol, 60*, 851-855.

Mangano, C., Scarano, A., Perrotti, V., Iezzi, G., & Piattelli, A. (2006). Maxillary sinus augmentation with a porous synthetic hydroxyapatite and bovine-derived hydroxyapatite: a comparative clinical and histologic study. *The International journal of oral & maxillofacial implants, 22*(6), 980-986.

Matsuura, A., Kubo, T., Doi, K., Hayashi, K., Morita, K., Yokota, R., . . . Akagawa, Y. (2009). Bone formation ability of carbonate apatite-collagen scaffolds with different carbonate contents. *Dental Materials Journal, 28*(2), 234-242.

Mauney, J. R., Volloch, V., & Kaplan, D. L. (2005). Role of adult mesenchymal stem cells in bone tissue engineering applications: current status and future prospects. *Tissue engineering, 11*(5-6), 787-802.

McNamara, L. E., McMurray, R. J., Biggs, M. J., Kantawong, F., Oreffo, R. O., & Dalby, M. J. (2010). Nanotopographical control of stem cell differentiation. *Journal of tissue engineering, 1*(1), 120623.

Meese, T. M., Hu, Y., Nowak, R. W., & Marra, K. G. (2002). Surface studies

of coated polymer microspheres and protein release from tissue-engineered scaffolds. *Journal of Biomaterials Science, Polymer Edition, 13*(2), 141-151.

Mehlhorn, A., Niemeyer, P., Kaschte, K., Muller, L., Finkenzeller, G., Hartl, D., . . . Schmal, H. (2007). Differential effects of BMP-2 and TGF-β1 on chondrogenic differentiation of adipose derived stem cells. *Cell Proliferation, 40*(6), 809-823.

Meinel, L., Karageorgiou, V., Fajardo, R., Snyder, B., Shinde-Patil, V., Zichner, L., . . . Vunjak-Novakovic, G. (2004). Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium flow. *Annals of biomedical engineering, 32*(1), 112-122.

Meng, J., Xiao, B., Zhang, Y., Liu, J., Xue, H., Lei, J., . . . Gu, N. (2013). Super-paramagnetic responsive nanofibrous scaffolds under static magnetic field enhance osteogenesis for bone repair in vivo. *Scientific Reports, 3*.

Michel, J., Penna, M., Kochen, J., Cheung, H., & Cheung, H. S. (2015). Recent Advances in Hydroxyapatite Scaffolds Containing Mesenchymal Stem Cells. *Stem Cells International, 2015*.

Mikos, A. G., & Temenoff, J. S. (2000). Formation of highly porous biodegradable scaffolds for tissue engineering. *Electronic Journal of Biotechnology, 3*(2), 23-24.

Mishra, V. K., Srivastava, S. K., Asthana, B. P., & Kumar, D. (2012). Structural and Spectroscopic Studies of Hydroxyapatite Nanorods Formed via Microwave-Assisted Synthesis Route. *Journal of the American Ceramic Society, 95*(9), 2709-2715.

Mohandes, F., Salavati-Niasari, M., Fereshteh, Z., & Fathi, M. (2014). Novel preparation of hydroxyapatite nanoparticles and nanorods with the aid of complexing agents. *Ceramics International, 40*(8), 12227-12233.

Mohanty, A., Misra, M., & Hinrichsen, G. (2000). Biofibres, biodegradable polymers and biocomposites: an overview. *Macromolecular materials and Engineering, 276*(1), 1-24.

Motskin, M., Wright, D., Muller, K., Kyle, N., Gard, T., Porter, A., & Skepper, J. (2009). Hydroxyapatite nano and microparticles: correlation of particle properties with cytotoxicity and biostability. *Biomaterials, 30*(19), 3307-3317.

Murray, P., Prewitz, M., Hopp, I., Wells, N., Zhang, H., Cooper, A., . . . Edgar, D. (2013). The self-renewal of mouse embryonic stem cells is regulated by cell–substratum adhesion and cell spreading. *The international journal of biochemistry & cell biology, 45*(11), 2698-2705.

Murugan, R., & Ramakrishna, S. (2004). Bioresorbable composite bone paste using polysaccharide based nano hydroxyapatite. *Biomaterials, 25*(17), 3829-3835.

Muruganandan, S., Roman, A., & Sinal, C. (2009). Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program. *Cellular and Molecular Life Sciences, 66*(2), 236-253.

Nöth, U., Osyczka, A. M., Tuli, R., Hickok, N. J., Danielson, K. G., & Tuan, R. S. (2002). Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. *Journal of Orthopaedic Research, 20*(5), 1060-1069.

Nair, M., Nancy, D., Krishnan, A. G., Anjusree, G., Vadukumpully, S., & Nair, S. V. (2015). Graphene oxide nanoflakes incorporated gelatin–hydroxyapatite scaffolds enhance osteogenic differentiation of human mesenchymal stem cells. *Nanotechnology, 26*(16), 161001.

Nakahara, H., Goldberg, V. M., & Caplan, A. I. (1991). Culture-expanded human periosteal-derived cells exhibit osteochondral potential in vivo. *Journal of Orthopaedic Research, 9*(4), 465-476.

Ng, F., Boucher, S., Koh, S., Sastry, K. S., Chase, L., Lakshmipathy, U., . . . Rao, M. S. (2008). PDGF, TGF-β, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways

important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. *Blood, 112*(2), 295-307.

Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Yamamoto, K., Adachi, E., . . . Watanabe, H. (2004). Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *New England Journal of Medicine, 351*(12), 1187-1196.

Nukavarapu, S. P., Kumbar, S. G., Merrell, J. G., & Laurencin, C. T. (2014). Electrospun polymeric nanofiber scaffolds for tissue regeneration. *Nanotechnology and Regenerative Engineering: The Scaffold*, 229.

Ooi, C., Hamdi, M., & Ramesh, S. (2007). Properties of hydroxyapatite produced by annealing of bovine bone. *Ceramics international, 33*(7), 1171-1177.

Orr, T., Villars, P., Mitchell, S., Hsu, H.-P., & Spector, M. (2001). Compressive properties of cancellous bone defects in a rabbit model treated with particles of natural bone mineral and synthetic hydroxyapatite. *Biomaterials, 22*(14), 1953-1959.

Osyczka, A. M., Nöth, U., Danielson, K. G., & Tuan, R. S. (2002). Different osteochondral potential of clonal cell lines derived from adult human trabecular bone. *Annals of the New York Academy of Sciences, 961*(1), 73-77.

Ozawa, M., Hattori, M., & Satake, K. (2007). *Waste management and application of fish bone hydroxyapatite for waste water treatment.* Paper presented at the Proceedings of International Symposium on EcoTopia Science.

Ozawa, M., & Kanahara, S. (2005). Removal of aqueous lead by fish-bone waste hydroxyapatite powder. *Journal of materials science, 40*(4), 1037-1038.

Ozawa, M., Satake, K., & Suzuki, R. (2003). Removal of aqueous chromium by fish bone waste originated hydroxyapatite. *Journal of Materials Science Letters, 22*(7), 513-514.

Ozawa, M., & Suzuki, S. (2002). Microstructural development of natural hydroxyapatite originated from fish-bone waste through heat treatment. *Journal of the American Ceramic Society, 85*(5), 1315-1317.

Padmanabhan, S. K., Salvatore, L., Gervaso, F., Catalano, M., Taurino, A., Sannino, A., & Licciulli, A. (2015). Synthesis and Characterization of Collagen Scaffolds Reinforced by Eggshell Derived Hydroxyapatite for Tissue Engineering. *Journal of Nanoscience and Nanotechnology, 15*(1), 504-509.

Pallela, R., Venkatesan, J., Janapala, V. R., & Kim, S. K. (2012). Biophysicochemical evaluation of chitosan-hydroxyapatite-marine sponge collagen composite for bone tissue engineering. *Journal of Biomedical Materials Research Part A, 100*(2), 486-495.

Palmer, L. C., Newcomb, C. J., Kaltz, S. R., Spoerke, E. D., & Stupp, S. I. (2008). Biomimetic systems for hydroxyapatite mineralization inspired by bone and enamel. *Chemical Reviews, 108*(11), 4754-4783.

Panda, N. N., Pramanik, K., & Sukla, L. B. (2014). Extraction and characterization of biocompatible hydroxyapatite from fresh water fish scales for tissue engineering scaffold. *Bioprocess and biosystems engineering, 37*(3), 433-440.

Park, S.-j., Lee, K. W., Lim, D.-S., & Lee, S. (2011). The sulfated polysaccharide fucoidan stimulates osteogenic differentiation of human adipose-derived stem cells. *Stem cells and development, 21*(12), 2204-2211.

Pazhanisamy, S. K. (2009). Stem cells, DNA damage, ageing and cancer. *Hematology/Oncology and Stem Cell Therapy, 2*(3), 375-384.

Pek, Y., Gao, S., Arshad, M., Leck, K.-J., & Ying, J. Y. (2008). Porous collagen-apatite nanocomposite foams as bone regeneration scaffolds. *Biomaterials, 29*(32), 4300-4305.

Peter, M., Ganesh, N., Selvamurugan, N., Nair, S., Furuike, T., Tamura, H., & Jayakumar, R. (2010). Preparation and characterization of chitosan–

gelatin/nanohydroxyapatite composite scaffolds for tissue engineering applications. *Carbohydrate Polymers, 80*(3), 687-694.

Petersen, B., Bowen, W., Patrene, K., Mars, W., Sullivan, A., Murase, N., . . . Goff, J. (1999). Bone marrow as a potential source of hepatic oval cells. *Science, 284*(5417), 1168-1170.

Phinney, D. G., & Prockop, D. J. (2007). Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views. *Stem Cells, 25*(11), 2896-2902.

Phipps, M. C., Clem, W. C., Grunda, J. M., Clines, G. A., & Bellis, S. L. (2012). Increasing the pore sizes of bone-mimetic electrospun scaffolds comprised of polycaprolactone, collagen I and hydroxyapatite to enhance cell infiltration. *Biomaterials, 33*(2), 524-534.

Piccirillo, C., Silva, M., Pullar, R., Braga da Cruz, I., Jorge, R., Pintado, M., & Castro, P. M. (2013). Extraction and characterisation of apatite-and tricalcium phosphate-based materials from cod fish bones. *Materials Science and Engineering: C, 33*(1), 103-110.

Piccirillo, C., Silva, M., Pullar, R., da Cruz, I. B., Jorge, R., Pintado, M., & Castro, P. M. (2013). Extraction and characterisation of apatite-and tricalcium phosphate-based materials from cod fish bones. *Materials Science and Engineering: C, 33*(1), 103-110.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., . . . Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science, 284*(5411), 143-147.

Planell, J. A., Best, S., Lacroix, D., & Merolli, A. (2009). *Bone repair biomaterials*: Elsevier.

Ponce, N. M., Pujol, C. A., Damonte, E. B., Flores, L., & Stortz, C. A. (2003). Fucoidans from the brown seaweed Adenocystis utricularis: extraction methods, antiviral activity and structural studies. *Carbohydrate Research, 338*(2), 153-165.

Prabakaran, K., & Rajeswari, S. (2006). Development of hydroxyapatite from

natural fish bone through heat treatment. *Trends Biomater Artif Organs, 20*, 20-23.

Pramanik, N., Tarafdar, A., & Pramanik, P. (2007). Capping agent-assisted synthesis of nanosized hydroxyapatite: comparative studies of their physicochemical properties. *Journal of materials processing technology, 184*(1), 131-138.

Qu-Petersen, Z., Deasy, B., Jankowski, R., Ikezawa, M., Cummins, J., Pruchnic, R., . . . Wernig, A. (2002). Identification of a novel population of muscle stem cells in mice potential for muscle regeneration. *The Journal of cell biology, 157*(5), 851-864.

Rahaman, M. N., Day, D. E., Sonny Bal, B., Fu, Q., Jung, S. B., Bonewald, L. F., & Tomsia, A. P. (2011). Bioactive glass in tissue engineering. *Acta Biomaterialia, 7*(6), 2355-2373.

Rajesh, R., Hariharasubramanian, A., & Ravichandran, Y. D. (2012). Chicken bone as a bioresource for the bioceramic (hydroxyapatite). *Phosphorus, Sulfur, and Silicon and the Related Elements, 187*(8), 914-925.

Reilly, T. M., Seldes, R., Luchetti, W., & Brighton, C. T. (1998). Similarities in the phenotypic expression of pericytes and bone cells. *Clinical Orthopaedics and Related Research, 346*, 95-103.

Rezwan, K., Chen, Q., Blaker, J., & Boccaccini, A. R. (2006). Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials, 27*(18), 3413-3431.

Riekstina, U., Cakstina, I., Parfejevs, V., Hoogduijn, M., Jankovskis, G., Muiznieks, I., . . . Ancans, J. (2009). Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. *Stem Cell Reviews and Reports, 5*(4), 378-386.

Riekstina, U., Muceniece, R., Cakstina, I., Muiznieks, I., & Ancans, J. (2008). Characterization of human skin-derived mesenchymal stem cell proliferation rate in different growth conditions. *Cytotechnology, 58*(3),

153-162.

Ringe, J., Leinhase, I., Stich, S., Loch, A., Neumann, K., Haisch, A., . . . Sittinger, M. (2008). Human mastoid periosteum-derived stem cells: promising candidates for skeletal tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine, 2*(2-3), 136-146.

Rodriguez, I. A., Madurantakam, P. A., McCool, J. M., Sell, S. A., Yang, H., Moon, P. C., & Bowlin, G. L. (2012). Mineralization potential of electrospun PDO-hydroxyapatite-fibrinogen blended scaffolds. *International journal of biomaterials, 2012*.

Romagnoli, C., & Brandi, M. L. (2014). Adipose mesenchymal stem cells in the field of bone tissue engineering. *World journal of stem cells, 6*(2), 144.

Romanov, Y. A., Darevskaya, A., Merzlikina, N., & Buravkova, L. (2005). Mesenchymal stem cells from human bone marrow and adipose tissue: isolation, characterization, and differentiation potentialities. *Bulletin of Experimental Biology and Medicine, 140*(1), 138-143.

Ruksudjarit, A., Pengpat, K., Rujijanagul, G., & Tunkasiri, T. (2008). Synthesis and characterization of nanocrystalline hydroxyapatite from natural bovine bone. *Current applied physics, 8*(3), 270-272.

Saboural, P., Chaubet, F., Rouzet, F., Al-Shoukr, F., Azzouna, R. B., Bouchemal, N., . . . Rolland, L. (2014). Purification of a Low Molecular Weight Fucoidan for SPECT Molecular Imaging of Myocardial Infarction. *Marine Drugs, 12*(9), 4851-4867.

Sadat-Shojai, M., Atai, M., & Nodehi, A. (2011). Design of experiments (DOE) for the optimization of hydrothermal synthesis of hydroxyapatite nanoparticles. *Journal of the Brazilian Chemical Society, 22*(3), 571-582.

Sakaguchi, Y., Sekiya, I., Yagishita, K., Ichinose, S., Shinomiya, K., & Muneta, T. (2004). Suspended cells from trabecular bone by collagenase digestion become virtually identical to mesenchymal stem cells obtained from marrow aspirates. *Blood, 104*(9), 2728-2735.

Salgado, A. J., Coutinho, O. P., & Reis, R. L. (2004). Bone tissue engineering:

state of the art and future trends. *Macromolecular Bioscience, 4*(8), 743-765.

Sanosh, K., Chu, M.-C., Balakrishnan, A., Kim, T., & Cho, S.-J. (2009). Utilization of biowaste eggshells to synthesize nanocrystalline hydroxyapatite powders. *Materials Letters, 63*(24), 2100-2102.

Sanosh, K., Chu, M.-C., Balakrishnan, A., Lee, Y.-J., Kim, T., & Cho, S.-J. (2009). Synthesis of nano hydroxyapatite powder that simulate teeth particle morphology and composition. *Current Applied Physics, 9*(6), 1459-1462.

Scherberich, A., Müller, A. M., Schäfer, D. J., Banfi, A., & Martin, I. (2010). Adipose tissue-derived progenitors for engineering osteogenic and vasculogenic grafts. *Journal of Cellular Physiology, 225*(2), 348-353.

Schlaubitz, S., Derkaoui, S. M., Marosa, L., Miraux, S., Renard, M., Catros, S., . . . Fricain, J.-C. (2014). Pullulan/dextran/nHA Macroporous Composite Beads for Bone Repair in a Femoral Condyle Defect in Rats.

Schneider, O. D., Loher, S., Brunner, T. J., Uebersax, L., Simonet, M., Grass, R. N., . . . Stark, W. J. (2008). Cotton wool-like nanocomposite biomaterials prepared by electrospinning: In vitro bioactivity and osteogenic differentiation of human mesenchymal stem cells. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 84*(2), 350-362.

Sekiya, I., Larson, B. L., Vuoristo, J. T., Cui, J. G., & Prockop, D. J. (2004). Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs). *Journal of Bone and Mineral Research, 19*(2), 256-264.

Sellgren, K. L., & Ma, T. (2012). Perfusion conditioning of hydroxyapatite–chitosan–gelatin scaffolds for bone tissue regeneration from human mesenchymal stem cells. *Journal of Tissue Engineering and Regenerative Medicine, 6*(1), 49-59.

Seong, J. M., Kim, B.-C., Park, J.-H., Kwon, I. K., Mantalaris, A., & Hwang, Y.-S. (2010). Stem cells in bone tissue engineering. *Biomedical*

Materials, 5(6), 062001.

Seyedjafari, E., Soleimani, M., Ghaemi, N., & Shabani, I. (2010). Nanohydroxyapatite-coated electrospun poly (l-lactide) nanofibers enhance osteogenic differentiation of stem cells and induce ectopic bone formation. *Biomacromolecules, 11*(11), 3118-3125.

Shea, L. D., Smiley, E., Bonadio, J., & Mooney, D. J. (1999). DNA delivery from polymer matrices for tissue engineering. *Nature Biotechnology, 17*(6), 551-554.

Sheridan, M., Shea, L., Peters, M., & Mooney, D. (2000). Bioabsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery. *Journal of Controlled Release, 64*(1), 91-102.

Shi, J., Votruba, A. R., Farokhzad, O. C., & Langer, R. (2010). Nanotechnology in drug delivery and tissue engineering: from discovery to applications. *Nano letters, 10*(9), 3223-3230.

Shimizu, K., Ito, A., Yoshida, T., Yamada, Y., Ueda, M., & Honda, H. (2007). Bone tissue engineering with human mesenchymal stem cell sheets constructed using magnetite nanoparticles and magnetic force. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 82*(2), 471-480.

Slater, B. J., Kwan, M. D., Gupta, D. M., Panetta, N. J., & Longaker, M. T. (2008). Mesenchymal cells for skeletal tissue engineering.

Sofronia, A. M., Baies, R., Anghel, E. M., Marinescu, C. A., & Tanasescu, S. (2014). Thermal and structural characterization of synthetic and natural nanocrystalline hydroxyapatite. *Materials Science and Engineering: C, 43*, 153-163.

Sommerfeldt, D., & Rubin, C. (2001). Biology of bone and how it orchestrates the form and function of the skeleton. *European Spine Journal, 10*(2), S86-S95.

Song, L., Young, N. J., Webb, N. E., & Tuan, R. S. (2005). Origin and characterization of multipotential mesenchymal stem cells derived from

adult human trabecular bone. *Stem cells and development, 14*(6), 712-721.

Sottile, V., Halleux, C., Bassilana, F., Keller, H., & Seuwen, K. (2002). Stem cell characteristics of human trabecular bone-derived cells. *Bone, 30*(5), 699-704.

Spitzer, R. S., Perka, C., Lindenhayn, K., & Zippel, H. (2002). Matrix engineering for osteogenic differentiation of rabbit periosteal cells using α-tricalcium phosphate particles in a three-dimensional fibrin culture. *Journal of Biomedical Materials Research, 59*(4), 690-696.

Stevens, M. M. (2008). Biomaterials for bone tissue engineering. *Materials today, 11*(5), 18-25.

Studeny, M., Marini, F. C., Champlin, R. E., Zompetta, C., Fidler, I. J., & Andreeff, M. (2002). Bone marrow-derived mesenchymal stem cells as vehicles for interferon-β delivery into tumors. *Cancer Research, 62*(13), 3603-3608.

Suntornnond, R., An, J., Yeong, W. Y., & Chua, C. K. (2015). Biodegradable Polymeric Films and Membranes Processing and Forming for Tissue Engineering. *Macromolecular Materials and Engineering*.

Swetha, M., Sahithi, K., Moorthi, A., Srinivasan, N., Ramasamy, K., & Selvamurugan, N. (2010). Biocomposites containing natural polymers and hydroxyapatite for bone tissue engineering. *International Journal of Biological Macromolecules, 47*(1), 1-4.

Tan, W., Krishnaraj, R., & Desai, T. A. (2001). Evaluation of nanostructured composite collagen-chitosan matrices for tissue engineering. *Tissue Engineering, 7*(2), 203-210.

Tang, M., Chen, W., Weir, M. D., Thein-Han, W., & Xu, H. H. (2012). Human embryonic stem cell encapsulation in alginate microbeads in macroporous calcium phosphate cement for bone tissue engineering. *Acta biomaterialia, 8*(9), 3436-3445.

Thein-Han, W., & Misra, R. (2009). Biomimetic chitosan–nanohydroxyapatite

composite scaffolds for bone tissue engineering. *Acta biomaterialia, 5*(4), 1182-1197.

Torroni, A. (2009). Engineered bone grafts and bone flaps for maxillofacial defects: state of the art. *Journal of Oral and Maxillofacial Surgery, 67*(5), 1121-1127.

Tower, J. (2012). Stress and stem cells. *Wiley Interdisciplinary Reviews: Developmental Biology, 1*(6), 789-802.

Tsai, W.-B., Chen, W.-T., Chien, H.-W., Kuo, W.-H., & Wang, M.-J. (2014). Poly (dopamine) coating to biodegradable polymers for bone tissue engineering. *Journal of Biomaterials Applications, 28*(6), 837-848.

Tseng, Y.-H., Kuo, C.-S., Li, Y.-Y., & Huang, C.-P. (2009). Polymer-assisted synthesis of hydroxyapatite nanoparticle. *Materials Science and Engineering: C, 29*(3), 819-822.

Tuan, R. S., Boland, G., & Tuli, R. (2003). Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Research and Therapy, 5*(1), 32-45.

Ueno, H., Mori, T., & Fujinaga, T. (2001). Topical formulations and wound healing applications of chitosan. *Advanced drug delivery reviews, 52*(2), 105-115.

Vacanti, C., & Vacanti, J. (1994). Bone and cartilage reconstruction with tissue engineering approaches. *Otolaryngologic Clinics of North America, 27*(1), 263-276.

Vacanti, C. A., & Bonassar, L. J. (1999). An overview of tissue engineered bone. *Clinical Orthopaedics and Related Research, 367*, S375-S381.

Vaculik, C., Schuster, C., Bauer, W., Iram, N., Pfisterer, K., Kramer, G., . . . Elbe-Bürger, A. (2012). Human dermis harbors distinct mesenchymal stromal cell subsets. *Journal of Investigative Dermatology, 132*, 563-574.

Van den Bos, C., Mosca, J., Winkles, J., Kerrigan, L., Burgess, W., & Marshak, D. (1997). Human mesenchymal stem cells respond to fibroblast growth

factors. *Human Cell, 10*(1), 45-50.

Venkatesan, J., Baboucarr, L., Manivasagan, P., Kang, K.-H., Chalisserry, E. P., Anil, S., . . . Kim, S. K. (2015). Isolation and Characterization of Nano-Hydroxyapatite from Salmon Fish Bone *Materials, 8*, Accepted.

Venkatesan, J., Bhatnagar, I., & Kim, S.-K. (2014). Chitosan-alginate biocomposite containing fucoidan for bone tissue engineering. *Marine Drugs, 12*(1), 300-316.

Venkatesan, J., & Kim, S.-K. (2010). Chitosan composites for bone tissue engineering—An overview. *Marine Drugs, 8*(8), 2252-2266.

Venkatesan, J., & Kim, S.-K. (2014). Nano-hydroxyapatite composite biomaterials for bone tissue engineering—a review. *Journal of Biomedical Nanotechnology, 10*(10), 3124-3140.

Venkatesan, J., & Kim, S. K. (2010). Effect of temperature on isolation and characterization of hydroxyapatite from tuna (Thunnus obesus) bone. *Materials, 3*(10), 4761-4772.

Venkatesan, J., & Kim, S. K. (2012). *Nanostructured hydroxyapatite-chitosan composite biomaterial for bone tissue engineering.* Paper presented at the Advanced Materials Research.

Venkatesan, J., Lowe, B., Anil, S., Manivasagan, P., Al Kheraif, A. A., Kang, K. H., & Kim, S. K. (2015). Seaweed polysaccharides and their potential biomedical applications. *Starch-Stärke, 67*(5-6), 381-390.

Venkatesan, J., Lowe, B., Manivasagan, P., Kang, K.-H., Chalisserry, E. P., Anil, S., . . . Kim, S.-K. (2015). Isolation and Characterization of Nano-Hydroxyapatite from Salmon Fish Bone. *Materials, 8*(8), 5426-5439.

Venkatesan, J., Lowe, B., Pallela, R., & Kim, S.-K. (2014). Chitosan-Based Polysaccharide Biomaterials *Polysaccharides* (pp. 1-13): Springer.

Venkatesan, J., Qian, Z.-J., Ryu, B., Kumar, N. A., & Kim, S.-K. (2011). Preparation and characterization of carbon nanotube-grafted-chitosan–natural hydroxyapatite composite for bone tissue engineering. *Carbohydrate polymers, 83*(2), 569-577.

Venkatesan, J., Qian, Z. J., Ryu, B., Ashok Kumar, N., & Kim, S. K. (2011). Preparation and characterization of carbon nanotube-grafted-chitosan - Natural hydroxyapatite composite for bone tissue engineering. *Carbohydrate polymers, 83*(2), 569-577. doi: 10.1016/j.carbpol.2010.08.019

Venkatesan, J., Qian, Z. J., Ryu, B., Thomas, N. V., & Kim, S. K. (2011). A comparative study of thermal calcination and an alkaline hydrolysis method in the isolation of hydroxyapatite from Thunnus obesus bone. *Biomedical Materials, 6*(3), 035003.

Venkatesan, J., Ryu, B., Sudha, P., & Kim, S.-K. (2012). Preparation and characterization of chitosan–carbon nanotube scaffolds for bone tissue engineering. *International Journal of Biological Macromolecules, 50*(2), 393-402.

Vozzi, G., Corallo, C., Carta, S., Fortina, M., Gattazzo, F., Galletti, M., & Giordano, N. (2014). Collagen-gelatin-genipin-hydroxyapatite composite scaffolds colonized by human primary osteoblasts are suitable for bone tissue engineering applications: In vitro evidences. *Journal of Biomedical Materials Research Part A, 102*(5), 1415-1421.

Wang, A., Liu, D., Yin, H., Wu, H., Wada, Y., Ren, M., . . . Xu, Y. (2007). Size-controlled synthesis of hydroxyapatite nanorods by chemical precipitation in the presence of organic modifiers. *Materials Science and Engineering: C, 27*(4), 865-869.

Wang, F., Zhang, L.-J., Wang, Y., & Liang, S.-Z. (2015). Feeder-Culture Bioreactors Inducing Osteogenic and Chondrogenic Differentiation of Human Mesenchymal Stem Cells on Polylactic Glycolic Acid/Tricalcium Phosphate (PLGA/TCP) Scaffolds. *Journal of Biomaterials and Tissue Engineering, 5*(7), 557-564.

Wang, F., Zhang, Y. C., Zhou, H., Guo, Y. C., & Su, X. X. (2014). Evaluation of in vitro and in vivo osteogenic differentiation of nano-hydroxyapatite/chitosan/poly (lactide-co-glycolide) scaffolds with

human umbilical cord mesenchymal stem cells. *Journal of Biomedical Materials Research Part A, 102*(3), 760-768.

Wang, H., Li, Y., Zuo, Y., Li, J., Ma, S., & Cheng, L. (2007). Biocompatibility and osteogenesis of biomimetic nano-hydroxyapatite/polyamide composite scaffolds for bone tissue engineering. *Biomaterials, 28*(22), 3338-3348.

Wang, J., He, C., Cheng, N., Yang, Q., Chen, M., You, L., & Zhang, Q. (2015). Bone Marrow Stem Cells Response to Collagen/Single-Wall Carbon Nanotubes-COOHs Nanocomposite Films with Transforming Growth Factor Beta 1. *Journal of Nanoscience and Nanotechnology, 15*(7), 4844-4850.

Wang, M., Yang, Y., Yang, D., Luo, F., Liang, W., Guo, S., & Xu, J. (2009). The immunomodulatory activity of human umbilical cord blood-derived mesenchymal stem cells in vitro. *Immunology, 126*(2), 220-232.

Wang, Q., Huang, C., Zeng, F., Xue, M., & Zhang, X. (2010). Activation of the Hh pathway in periosteum-derived mesenchymal stem cells induces bone formation in vivo: implication for postnatal bone repair. *The American journal of pathology, 177*(6), 3100-3111.

Wang, X., Li, Y., Wei, J., & De Groot, K. (2002). Development of biomimetic nano-hydroxyapatite/poly (hexamethylene adipamide) composites. *Biomaterials, 23*(24), 4787-4791.

Watson, B. M., Kasper, F. K., Engel, P. S., & Mikos, A. G. (2014). Synthesis and Characterization of Injectable, Biodegradable, Phosphate-Containing, Chemically Cross-Linkable, Thermoresponsive Macromers for Bone Tissue Engineering. *Biomacromolecules, 15*(5), 1788-1796.

Wei, G., & Ma, P. X. (2004). Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering. *Biomaterials, 25*(19), 4749-4757.

Whang, K., Goldstick, T. K., & Healy, K. E. (2000). A biodegradable polymer scaffold for delivery of osteotropic factors. *Biomaterials, 21*(24), 2545-

2551.

Woo, K. M., Jun, J.-H., Chen, V. J., Seo, J., Baek, J.-H., Ryoo, H.-M., . . . Ma, P. X. (2007). Nano-fibrous scaffolding promotes osteoblast differentiation and biomineralization. *Biomaterials, 28*(2), 335-343.

Worster, A. A., Nixon, A. J., Brower-Toland, B. D., & Williams, J. (2000). Effect of transforming growth factor β1 on chondrogenic differentiation of cultured equine mesenchymal stem cells. *American Journal of Veterinary Research, 61*(9), 1003-1010.

Wu, S., Liu, X., Yeung, K. W., Liu, C., & Yang, X. (2014). Biomimetic porous scaffolds for bone tissue engineering. *Materials Science and Engineering: R: Reports, 80*, 1-36.

Xia, Z., Yu, X., Jiang, X., Brody, H. D., Rowe, D. W., & Wei, M. (2013). Fabrication and characterization of biomimetic collagen–apatite scaffolds with tunable structures for bone tissue engineering. *Acta Biomaterialia, 9*(7), 7308-7319.

Xu, H. H., Weir, M. D., & Simon, C. G. (2008). Injectable and strong nano-apatite scaffolds for cell/growth factor delivery and bone regeneration. *Dental Materials, 24*(9), 1212-1222.

Xu, J., Khor, K. A., Dong, Z., Gu, Y., Kumar, R., & Cheang, P. (2004). Preparation and characterization of nano-sized hydroxyapatite powders produced in a radio frequency (rf) thermal plasma. *Materials Science and Engineering: A, 374*(1), 101-108.

Yoshimura, H., Muneta, T., Nimura, A., Yokoyama, A., Koga, H., & Sekiya, I. (2007). Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell and Tissue Research, 327*(3), 449-462.

Young, H. E., Steele, T. A., Bray, R. A., Hudson, J., Floyd, J. A., Hawkins, K., . . . Cuzzourt, J. (2001). Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. *The Anatomical*

Record, 264(1), 51-62.

Younger, E. M., & Chapman, M. W. (1989). Morbidity at bone graft donor sites. *Journal of Orthopaedic Trauma, 3*(3), 192-195.

Yu, B., Zhang, Y., Li, X., Wang, Q., Ouyang, Y., Xia, Y., . . . Fan, Y. (2013). The use of injectable Chitosan/Nanohydroxyapatite/collagen composites with bone marrow mesenchymal stem cells to promote ectopic bone formation in vivo. *Journal of Nanomaterials, 2013*, 11.

Yunoki, S., Sugiura, H., Ikoma, T., Kondo, E., Yasuda, K., & Tanaka, J. (2011). Effects of increased collagen-matrix density on the mechanical properties and in vivo absorbability of hydroxyapatite–collagen composites as artificial bone materials. *Biomedical Materials, 6*(1), 015012.

Zainon, I., Alwi, N., Abidin, M., Haniza, H., Ahmad, M., & Ramli, A. (2012). Physicochemical Properties of Hydroxyapatite Extracted from Fish Scales. *Advanced Materials Research, 545*, 235-239.

Zannettino, A., Paton, S., Arthur, A., Khor, F., Itescu, S., Gimble, J., & Gronthos, S. (2008). Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. *Journal of Cellular Physiology, 214*(2), 413-421.

Zhang, H.-b., Zhou, K.-c., Li, Z.-y., & Huang, S.-p. (2009). Plate-like hydroxyapatite nanoparticles synthesized by the hydrothermal method. *Journal of Physics and Chemistry of Solids, 70*(1), 243-248.

Zhang, S., & Gonsalves, K. (1997). Preparation and characterization of thermally stable nanohydroxyapatite. *Journal of Materials Science: Materials in Medicine, 8*(1), 25-28.

Zhang, S., Prabhakaran, M. P., Qin, X., & Ramakrishna, S. (2015a). Biocomposite scaffolds for bone regeneration: Role of chitosan and hydroxyapatite within poly-3-hydroxybutyrate-co-3-hydroxyvalerate on mechanical properties and in vitro evaluation. *Journal of the mechanical behavior of biomedical materials, 51*, 88-98.

Zhang, S., Prabhakaran, M. P., Qin, X., & Ramakrishna, S. (2015b). Poly-3-hydroxybutyrate-co-3-hydroxyvalerate containing scaffolds and their integration with osteoblasts as a model for bone tissue engineering. *Journal of Biomaterials Applications, 29*(10), 1394-1406.

Zhang, T., Wu, X., Pu, X., Huang, H., Zhang, Y., Li, M., & Xia, H. (2015). Comparative Study of Different Coatings on Magnesium Alloys: The Effect on MSC Proliferation and Osteogenic Differentiation. *Journal of Biomaterials and Tissue Engineering, 5*(7), 579-585.

Zhang, Y., Venugopal, J. R., El-Turki, A., Ramakrishna, S., Su, B., & Lim, C. T. (2008). Electrospun biomimetic nanocomposite nanofibers of hydroxyapatite/chitosan for bone tissue engineering. *Biomaterials, 29*(32), 4314-4322.

Zhao, L., Weir, M. D., & Xu, H. H. (2010). An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering. *Biomaterials, 31*(25), 6502-6510.

Zhao, Z., Liao, L., Cao, Y., Jiang, X., & Zhao, R. (2005). Establishment and properties of fetal dermis-derived mesenchymal stem cell lines: plasticity in vitro and hematopoietic protection in vivo. *Bone Marrow Transplantation, 36*(4), 355-365.

Zheng, Y.-x., Ringe, J., Liang, Z., Loch, A., Chen, L., & Sittinger, M. (2006). Osteogenic potential of human periosteum-derived progenitor cells in PLGA scaffold using allogeneic serum. *Journal of Zhejiang University SCIENCE B, 7*(10), 817-824.

Zhong, M.-L., Li, M., & Li, L.-H. (2015). Effects of different lengths of Ti nanorods topography on mesenchymal stem cell growth and proliferation. *Journal of Nanoscience and Nanotechnology, 15*(5), 3893-3899.

Zhou, H., & Lee, J. (2011). Nanoscale hydroxyapatite particles for bone tissue engineering. *Acta biomaterialia, 7*(7), 2769-2781.

Zhou, J., Zhang, X., Chen, J., Zeng, S., & De Groot, K. (1993). High

temperature characteristics of synthetic hydroxyapatite. *Journal of Materials Science: Materials in Medicine, 4*(1), 83-85.

Zhou, P., Cheng, X., Xia, Y., Wang, P., Zou, K., Xu, S., & Du, J. (2014). Organic/Inorganic Composite Membranes Based on Poly (L-Lactic-co-Glycolic Acid) and Mesoporous Silica for Effective Bone Tissue Engineering. *ACS applied materials & interfaces, 6*(23), 20895-20903.

Ziegler, J., Mayr-Wohlfart, U., Kessler, S., Breitig, D., & Günther, K. P. (2002). Adsorption and release properties of growth factors from biodegradable implants. *Journal of Biomedical Materials Research, 59*(3), 422-428.

Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., . . . Hedrick, M. H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Engineering, 7*(2), 211-228.

Zvaifler, N. J., Marinova-Mutafchieva, L., Adams, G., Edwards, C. J., Moss, J., Burger, J. A., & Maini, R. N. (2000). Mesenchymal precursor cells in the blood of normal individuals. *Arthritis research, 2*(6), 477-488.

5.1. Acknowledgement

First of all, I would like to extend my profound gratitude and sincere thanks to Prof. Se-Kwon Kim for availing me the unique opportunity to work in his research group providing a highly sophisticated Laboratory environment for cutting-edge research especially in my research area. He has given me all the support, guidance and encouragement during the course of my entire research program sharing his terrific research experiences.

Secondly, my sincere thanks and gratitude also goes Dr. Jayachandran Venkatesan for introducing me to this research field. His mentorship, guidance and support has earn me success in my research. A highly motivated scholar, Dr. Venkatesan's work ethics and research experiences are indeed insurmountable. He worked diligently to preparing me in all frontiers to becoming an independent researcher.

My thanks and appreciation also goes to Dr. Ramjee Pallela for this guidance and encouragement. He has been a sentinel to me-supporting me to realizing my research ambitions since I was an undergraduate student. Even in his busy schedule he would

attend to me and provide answers to my questions and as well share research experiences. And his wife Dr. Ira Bhatnagar for all the support. I thank them for everything they have done for me.

To my colleagues Sea Hun Oh, Soyeon Kim, Ding Yishan, Moch Syaiful Alam thank you so much for being nice people. I will always remember the beautiful moments were all shared together. You have enhanced my learning and research process at the research center by being supportive and kind to me. To Dr. Young-Sang Kim and Dr. Kyong-Hwa Kang you are incredibly nice people and I will always live to remember you. I appreciate all the support and assistance you have rendered me during the course of my study. Miyong Kim, Sohn Chongsun, Park Yong Woo, Dr. Kim and Mr. Moon thank you all for helping me adapt to Korean life and culture. I deeply appreciate your friendship.

To my family in Gambia and friends abroad, I want to thank you for all the support and prayers. To my uncles, grandmother, aunts, cousins, brothers, sisters you all occupy special places in my heart and I will never relent in my efforts to keep putting smiles in your beautiful faces. Many special thanks goes to my uncle Bai, for all his support to me.

Finally, I want to thank my mother Isatou Cham for she has been there for me since day one bearing the burden of motherhood with love and a high sense of trust and hope all times. I pray for her the best of health, long live and strength. To my late father Omar Lowe, I hold you close within my heart and there you will remain to walk with me throughout my life until we meet again. To my brothers Bai and Jim and my sisters Fatou, Ida and Haddy you are all special to me and I pray each new day bring a feeling of excitement, joy, smile and wonderful sense of expectation.

Last but not the least, I want to thank the almighty Allah-the omnipotent for giving me the opportunity and strength to successfully complete my research.

I hope this work would be one the great number of research engagements I wish to undertake in future. I hope this piece of work would also provide a platform for future research in the field of bone tissue engineering.

Peace be upon you!

Baboucarr Lowe