Optimization of Hydroxyapatite β-Tricalcium Phosphate – Fibrin Constructs for Human Mesenchymal Stem Cell Proliferation and Mechanical Strength

An Internship/ Team Project Report Presented to

the Faculty of the Biology Program

California State University Channel Islands

In Partial Fulfillment of

the Requirements for the Degree of

Master of Science in Biotechnology and Bioinformatics

By

Roxana Fard

September 25, 2010

Optimization of Hydroxyapatite β-Tricalcium Phosphate – Fibrin Constructs for Human Mesenchymal Stem Cell Proliferation and Mechanical Strength

An Internship/ Team Project Report Presented to

the Faculty of the Biology Program

California State University Channel Islands

By

Roxana Fard

September 25, 2010

APPROVED BY THE BIOLOGY PROGRAM

10/25/2010

Dr. Ching-Hua Wang Date

APPROVED BY THE UNIVERSITY

10/25/10

Dr. Gary A. Berg Date

Acknowledgement

First and foremost I would like to thank Dr. Bill Tawil for his mentorship and support over the past year at UCLA. I would also like to thank Dr. Ben Wu for allowing me the privilege to conduct my research in his laboratory. A special thanks to Chase Linsley for his guidance in experimenting and the analysis of data.

I would also like to thank the faculty at CSUCI for giving me the opportunity to embark on this year long adventure. Thank you Dr Chin-Hua Wang for establishing this program and to her hard work in organizing everything from the ground up. Also, thanks to Jennifer Ormond for her hard work and dedication to the program. I truly appreciate it.

Lasty, a big thanks my parents for always supporting me in everything.

This work is partially supported by the Bridges to Stem Cell Research Grant TB1-01177 awarded to Dr. Ching-Hua Wang at California State University Channel Islands by the California Institute for Regenerative Medicine.

Abstract

Bone graft substitutes are commonly used as an alloplastic source for complex bone repair. Human mesenchymal stem cells (hMSCs) have become an idealistic source for bone repair and regeneration due to their potential to differentiate into osteogenic precursors. The purpose of engineering synthetic bone grafts it to successfully find a substitute that is biocompatible, bioresorbable, and has osteoconductive characteristics. The purpose of this study is to construct a bone biocomposite with an optimal amount of biphasic hydroxyapatite/β-tricalcium phosphate (HA-TCP) powder to promote hMSC proliferation with sufficient mechanical stiffness.Results have indicated an increase in metabolic proliferation over a 2-week time period. The constructs seeded with hMSCs exhibited a 3 to 9 fold or greater increase in proliferation depending on the formulation of the construct. This work demonstrates that higher volumes of HA-TCP promote hMSC proliferation in the constructs while maintaining sufficient mechanical stiffness. Optimizing the components of the scaffolds will allow for the most innovative biomimetic bone composite for mesenchymal stem cell differentiation into osteoblasts in an *in vivo* model.

Table of Contents

Introduction

Bone Defects and Repair

Unlike most organs in the body, bone tissue has the capacity to regenerate after minor breaks and fractures. Bone healing is a complex physiological process involving a cascade of events that lead to the repair of fractures and breaks [1]. The first stage of the bone repair cascade is inflammation and the formation of a hematoma 1-3 days following the fracture. The fracture callus forms with the recruitment of chondroblasts and osteoblasts to the fracture site a couple weeks after the fracture. 2-6 months after the fracture the bone is in the remodeling phase where the bone reshapes into its original form. Severe defects and other complications can prevent bone from regeneration. Tumor resection, trauma, congenital abnormalities, bone cysts, and periodontal disease are examples of major causes of bone defects [2,3]. The current treatment options for critical bone defects include the use of autografts, allografts, xenografts, and alloplastic grafts. Typically derived from the patients own iliac crest, autografts require an additionally surgery with the potential of donor site morbidity. Allografts are derived from deceased donors and are slightly less invasive because they only require a single surgery to repair the damaged bone. These however carry the risk of possible pathogenic transmission. Xenografts are derived from animals and have similar limitations to autografts and allografts. Increasing in popularity are the man-made alloplastic grafts. Biomaterials such as calcium phosphates and hydroxyapatite can provide the biocompatibility needed for successful resorption into bone while maintaining the environment necessary for cell proliferation and differentiation [4].

Scaffolds for cell culture

3-D scaffold structures have provided a more accurate approach in tissue engineering because they can be engineered to closely mimic the microenvironment present in the human body [5]. They are designed functionally, structurally, and mechanically comparable to the tissues in which they replace [6]. The fundamental properties to consider when formulating an effective bone biocomposite are: biocompatibility, resorbability, osteoinductivity, and osteoconductivity [7]. Biocompatibility in tissue engineering refers to the ability of a construct to support cellular activity without detrimental effects [8]. Resorbability is key in order for the components of the biocomposite to degrade and become incorporated into the damaged bone. Osteoinductivity is the ability for the surrounding environment to induce cells into bone formation. Osteoconductivity refers to the ability of the graft to support the formation of new bone while allowing attachment of new osteoblast and osteoprogenitor cells [9]. Fibrin scaffolds have become desirable in tissue engineering research because it mimics the final step in the wound healing process by creating a gel clot that supports cell adhesion, migration, and differentiation [10]. Fibrin is naturally present in the wound-healing cascade and functions as a mesh of extracellular matrix proteins to cover the wound site [11]. Many studies have shown that given the right properties, fibrin scaffolds can support the proliferation of numerous cell types. Additionally, fibrin is easily purified from blood plasma, is biocompatible, and easily degraded *in vitro* and *in vivo.* Although fibrin is an excellent choice for the proliferation of numerous types of cells, it is

mechanically too weak to be used independently as bone filler. Thus phosphate ceramics are used as a favorable component for scaffolds used in bone repair and regeneration.

B-Tricalcium Phosphate

When a bone is unable to heal itself due to a serious bone defect, bone grafts are used to fill the damaged area. Synthetic bone grafting materials such as calcium phosphate ceramics are commonly used as bone substitutes in clinical and biomedical applications in lieu of autografts and allografts. Ceramics have favorable mechanical properties because they are stiffer than other soft biomaterials and have been shown to promote mineralization in *in vitro* studies [12]. The advantage of using ß-tricalcium phosphate is that it is compatible with bone tissue and is easily degraded in the human body. Some experiments have shown that although they possess osteoconductive properties, they lack osteogenicity for repairing large defects [13]. Thus additional experiments are being conducted with composite materials for increased osteoinducrivity and osteogenicity.

Hydroxyapatite

Macroporous hydroxyapatite (HA) is a natural calcium phosphate mineral present in the bones and teeth of mammalian species [14]. HA is often used with medical implants because of the osteoinductive properties it possesses. HA chemically binds directly to bone [15] and is highly favorable for the attachment of osteoblast cells [16]. Similar to ß-tricalcium phosphate, it is biocompatible with bone tissue but is far less soluble. In healthy bone regeneration hydroxyapatite recruits osteoprogenitor cells to produce growth factors, which in turn recruit mesenchymal stem cells (MSCs) for differentiation

into bone cells [17]. These characteristics make HA desirable for use in bone grafting procedures.

Mesenchymal Stem cells – Bone regeneration

Human mesenchymal stem cells (hMSCs) have become an idealistic source for bone repair and regeneration due to their potential to differentiate into osteogenic precursors. Depending on the culture conditions these multipotent, bone marrow-derived cells are capable of differentiating into osteoblasts, chondroblasts, adipocytes, or fibroblasts [18,19]. More studies are starting to show that MSCs can be induced into differentiation with their surrounding environment [20], thus stressing the importance of ECM components.

Objective

The objective of the following experiments is to optimize a biphasic ceramic with human mesenchymal stem cells to promote proliferation with sufficient mechanical stiffness. The overall goal with this research is to develop an implantable ceramic that includes growth factors encapsulated in fibrin and/or collagen beads with sustained release to aid in the proliferation and differentiation of hMSCs into osteoblast cells.

Materials and Methods

Hydroxyapatite coated ß-tricalcium phosphate

The microporous and macroporous HA/TCP granules are 60% hydroxyapatite (HA) and 40% β-Tricalcium Phosphate (TCP) that are 1-2 mm in size. The granules are grated to

100-500 microns in size to allow for a homogenous construct. For each formulation, the ceramic powder was individually weighed and sterilized. All samples were sterilized for 60 minutes at 121 °C prior to use. Aseptic technique was used to ensure that all ceramic materials remained sterile prior to experimentation. Baxter Healthcare Corporation generously donated the biphasic HA-TCP for this study.

Fibrin Matrix

Human Sealer Protein Concentrate (fibrinogen component) was reconstituted in bovine Fibrinolysis Inhibitor Solution and diluted in calcium chloride solution to appropriate concentrations as per experiment. Human thrombin was reconstituted in calcium chloride to produce the thrombin solution. The thrombin solution was further diluted in calcium chloride to appropriate concentrations per experiment. Baxter Healthcare Corporation donated the fibrin matrix components for this study.

Cell Lines

Human mesenchymal stem cells (hMSCs; Lonza, Basel, Switzerland) were propagated in Mesenchymal Stem Cell Basal medium (MSCGM, Lonza) and maintained in low glucose Dulbecco's Modified Eagles Medium (DMEM, Cellgro) with 10% fetal bovine serum (FBS, Omega Scientific, Inc; Tarzana, CA). Normal human dermal fibroblasts (NHDFs; American Type Culture Collection, Manassas, VA) were propagated in high glucose DMEM (Lonza) with 10% fetal bovine serum (FBS; Omega Scientific, Inc). Normal human osteoblasts (NHObs; Lonza) were propagated in osteoblast growth medium (OGM, Lonza) containing 10% fetal bovine serum (FBS, Lonza). Mouse pre-osteoblasts

(MC3T3s; American Type Culture Collection) were propagated in Alpha Modified Eagles Medium (α-MEM; Cellgro) with 10% fetal bovine serum (FBS, Omega Scientific, Inc). All cells were maintained at 37 °C in a 5% CO2 incubator (VWR, Bridgeport, NJ).

Ceramic gel optimization

In order to determine the various ratios of the ceramic, initial optimization of the fibrin-HA-TCP scaffold needed to be assessed to determine pliability. To ensure sufficient mixing, the HA-TCP can only be combined with one component of the fibrin matrix. After mixing, the second component is added and allowed to polymerize into the fibrin-HA-TCP biphasic ceramic. For this optimization ß-tricalcium phosphate (TCP) was used in place of the hydroxyapatite coated tricalcium phosphate. Volume-to-volume ratios of the fibrinogen to ceramic powder were measured at 4:1, 2:1. 1:1, 1:2, and 1:4 were tested. The 4:1 fibrinogen-to-ceramic mixture was slightly pasty and polymerized quickly with the addition of thrombin. The 2:1 mixture was clumpy and formed grainy clusters with the addition of the thrombin. For the 1:1, 1:2, and 1:4 fibrinogen-to-TCP mixtures, the ceramic had completely absorbed the fibrinogen and had remained in the powdered form after the addition of the thrombin into the wells. This optimization was key in indicating that the experimental constructs could not exceed 30% ceramic by volume.

Scaffold Fabrication

The scaffolds were constructed by mixing the pre-weighed HA-TCP, fibrinogen, and cells inside a syringe. The contents were swirled around to ensure even mixing and increased homogeneity. The thrombin was drawn up from the well plate into the syringe

and the contents of the syringe were depressed into the well. The scaffolds were allowed at least 15 minutes to set before adding medium to the wells.

Alamar Blue Assay

The alamarBlue® assay is a non-toxic, colorimentric assay that rapidly detects metabolic proliferation by detecting oxidation through respiration. The REDOX indicator in the reaction is specifically selected to undergo colorimetric change and fluoresce without interrupting the electron transport chain (Appendix 1 Table 1). An unreduced alamarBlue® reagent is dark blue in color and gradually changes to a shade of pink as it oxidizes. A fully reduced sample will be hot pink in color.

Constructs were washed in 500 µl HBSS buffer (Cellgro) for 5 minutes. A 1:10 dilution of alamarBlue® reagent (ABdSerotec) was made with medium and 500 µl was added to each construct. The samples were incubated at 37 °C for 6 hours. After the incubation was complete, the fluorescence intensity was measured with a Tecan plate reader and Magellan software. The equation to calculate the percent reduction of alamarBlue® is as follows:

$$\text{\% reduction of alamarBlue®} = \frac{\text{FI of test agent - FI untreated control}}{\text{FI of 100\% reduced alamarBlue® - FI untreated control}} \times 100$$

Where: FI = Fluorescent Intensity at 595nm emission (5353nm excitation)

Mechanical Testing

Compression testing is used to determine the mechanical behavior of materials. Young's Modulus uses Hookes's Law to measure the uniaxial stress over uniaxial strain. Hooke's Law directly states that the strain is directly proportional to the stress resulting in a linear

model. Other factors include Poisson's Ratio, which measures the stress of the material opposite from which it is compressed.

Mechanical compression testing was done with the Instron 3365 Universal Testing Machine using a 2.5N load cell. Instron compression data yields the stress-strain curve of the ceramic scaffold as it is compressed. After the removal of the excess media from the wells, the constructs were compressed with a flat-ended metal rod at a controlled displacement of 5 mm/min until a final displacement of 1 mm was reached (Appendix 1 Figure 1).

Results

Proliferation – Constructs with variations in HA-TCP volume

Testing was done on constructs containing fibroblasts (NHDFs), human mesenchymal stem cells (hMSCs), and osteoblasts (Hobs). Also, as a negative control, constructs without ceramic powders were tested. 6 hour Alamar Blue results indicate an increase in metabolic proliferation over a 2-week period for the fibroblast and osteoblast control constructs and the constructs with the hMSCs (Fig 1 A-C). The fibroblast constructs show a 3 to 3.5-fold increase in proliferation by day 14. The constructs seeded with hMSCs show little increase in proliferation on day 7 but by day 14 the constructs seeded with hMSCs exhibit an 8 to 9-fold increase in proliferation (Fig 1 B). The hMSC constructs that contained 20% HA-TCP by volume showed the greatest proliferation with a 9-fold increase. The osteoblast constructs showed a 2 to 3-fold increase in proliferation by day 14 (Fig 1 C). There was no significant difference observed in proliferation observed between most of the constructs containing the ceramic powder and the

constructs without. This indicates that the HA-TCP is biocompatible without having toxic effects on the cells. The osteoblasts in the fibrin gel alone were the only constructs that demonstrated lower proliferation than those without the HA-TCP powder. Degradation of the constructs was also observed in the constructs seeded with hMSCs. The most severe degradation was observed in the constructs with 20% HA-TCP ceramic. These constructs had degraded about 30-40% of their original height and diameter.

Mechanical Stiffness – Constructs with variations in HA-TCP volume

Instron compression testing was done to analyze the stiffness of the HA-TCP constructs (Fig 2). Results indicated that there was little to no change in stiffness in the fibroblast and osteoblast constructs by day 14 (Fig 2 A, C). The constructs seeded with hMSCs however, had a 1.4 to 1.6-fold increase in stiffness by day 14 (Fig 2 B). The fibrin gels without the ceramic also showed little to no change between day 1 and day 14. The increase in stiffness observed is the result of the HA-TCP that remained after the degradation of the fibrin. As noted in the results for the proliferation assay, several of the constructs had degraded 30-80% of the original size. The fibroblast and osteoblast constructs showed little to no degradation in size and shape.

Discussion

Having an optimally designed scaffold for hMSC proliferation is key in the tissue engineering strategy for bone repair and regeneration. An important aspect of this research is to create a construct with higher proliferation to reduce the need for high amounts growth factors added to future models. In order to create the most optimal

scaffold for hMSC proliferation, it is necessary to determine what the optimal volume of HA-TCP and fibrinogen concentrations are. One of the characteristics of the hydroxyapatite in the bioceramic is that it promotes cell growth into osteoblasts, which is consistent with the proliferation data shown in this experiment. Results from the first experiment demonstrate that higher volumes of HA-TCP promote hMSC proliferation in the constructs while maintaining sufficient mechanical stiffness. However, due to the settling of the HA-TCP powder on the bottom of the wells, constructs with 10% HA-TCP was used for subsequent experiments. One of the greatest challenges affecting the mechanical properties was the homogeneity of the ceramic within the construct. Multiple attempts were made at finding an optimal method of mixing the components of the construct before it polymerized into a solid. Since the HA-TCP is a heavier component of the construct, some of it settled along the bottom of the wells and created a short layer of ceramic. This is consistent with the current knowledge of hydroxyapatite properties. Since it is less soluble than calcium phosphates it created a challenge in having a completely homogeneous construct.

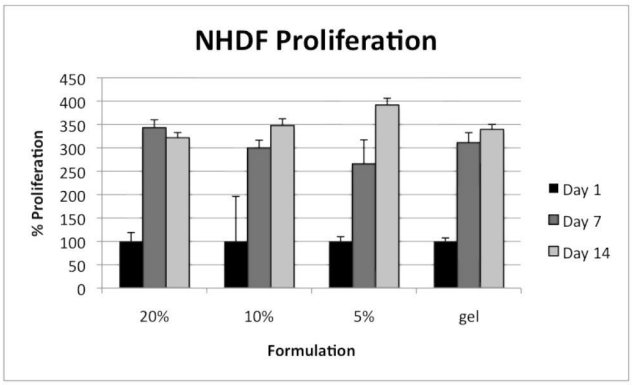
Further work to be carried out on this study is additional testing of the constructs with the variations in fibrinogen as well as changes in the thrombin concentration. Optimizing the components of the scaffolds will allow for the most innovative biomimetic bone composite for mesenchymal stem cell differentiation into osteoblasts in an *in vivo* model. This will fully optimize all components of the biphasic ceramic before the addition of growth factors into the model. Growth factors will enhance the properties of the scaffold while promoting osteogenic differentiation of the hMSCs into osteoblasts.

Summary

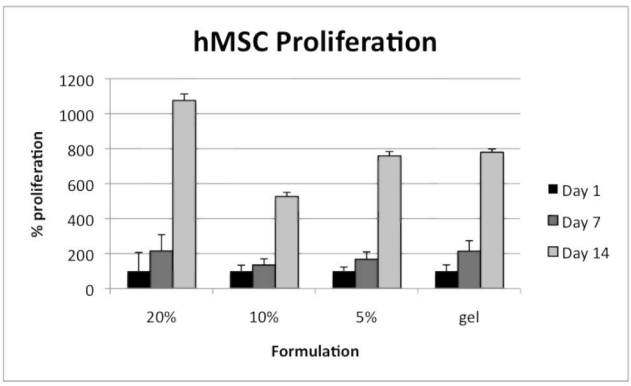
This experiment has provided useful information in the optimization of biphasic calcium ceramic scaffolds for hMSC proliferation and stiffness assessment. Here we tested variations in the formulation of the HA-TCP constructs. The results indicated an increase in proliferation as well as an increase in stiffness in constructs with higher volumes of HA-TCP. As the first step in the optimization process, it is key to develop the correct formulation. Further testing will be conducted where the concentrations of fibrinogen and thrombin. It is important that we obtain similar results to be able to come to a definite conclusion so we can move forward in further optimizing the fibrin-HA-TCP construct.

Figure 1. 6 hour alamarBlue® assay to determine metabolic proliferation of F-HA-TCP constructs with variations in HA-TCP. (A) Shows the fibroblast proliferation in the ceramic and gel constructs. (B) Shows the mesenchymal stem cell proliferation in the ceramic and gel constructs. (C) Shows the osteoblast proliferation in the ceramic and gel constructs.

A



B



C

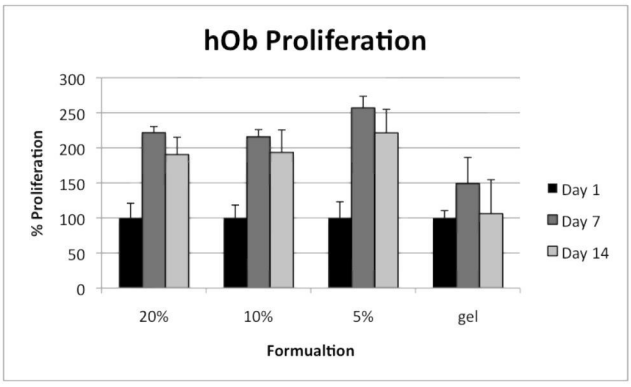
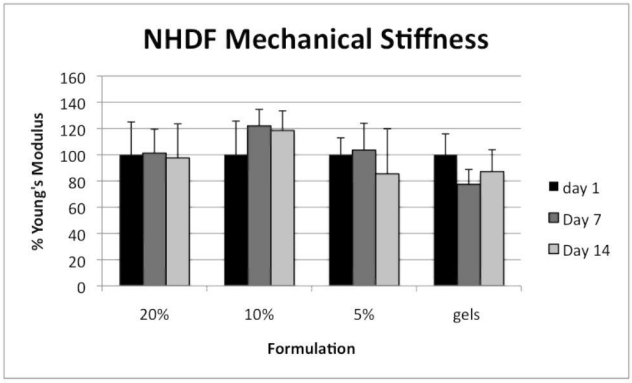
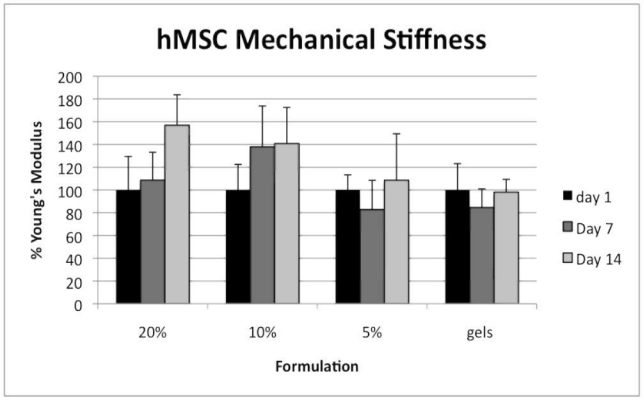


Figure 2. Mechanical Stiffness of constructs with variations in HA-TCP volume. (A) Shows the Instron Compression testing on fibroblast (NHDF) ceramic and gel constructs. (B) Shows the Instron Compression testing on mesenchymal stem cell (hMSC) ceramic and gel constructs. (C) Shows the Instron Compression testing on osteoblast (Hob) ceramic and gel constructs

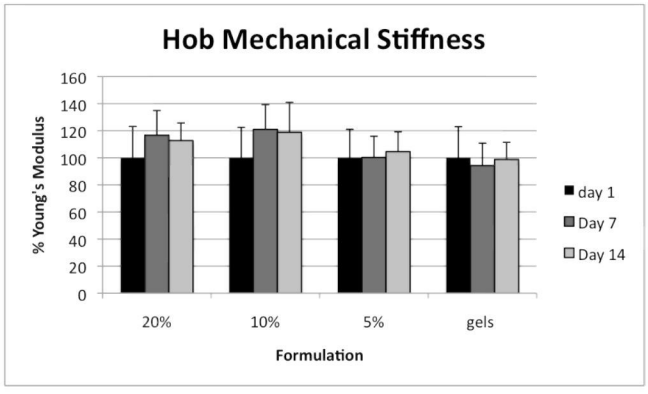
A



B



C



Literature Cited

1. Einhorn T.A. (1998) The cell and molecular biology of fracture healing. *Clinical Orthopaedics and Related Research.* S7-S21

2. Matsumoto G. et al. (2009) Enhanced Regeneration of Critical Bone Defects Using a Biodegradable Gelatin Sponge and ß-Tricalcium Phosphate with Bone Morphogenic Protein-2. *J of Biomaterials Applications*. 24:327-342

3. Luginbuehl V, Meinel L, Merkle H, Gander B. (2004) Localized Delivery of growth factors for bone repair. *E. J. of Pharmaceutics and Biopharmaceutics* 58: 197-208

4. Hokugo A, Takamoto T, Tabata Y. (2005) Preparation of hybrid scaffold from fibrin and biodegradable polymer fiber. *Biomaterials.* 27: 61-67

5. Dawson E, Mapili G, Erickson K, Taqvi S, Roy K. (2007) Biomaterials for stem cell differentiation. *Advanced Drug Delivery*. 60: 215-228

6. Puppi D, Chielllini F, Piras A.M, Chiellini E. (2010) Polymeric materials for bone and cartilage repair. *Progress in Polymer Science*. 35: 403-440

7. Kwon S, Jun Y, Hong S, Kim H. (2002) Synthesis and dissolution behavior of ß-TCP and HA/ß-TCP composite powders. *J of European Ceramic Society* 23:1039-1045

8. Dorozhkin S. (2009) Bioceramics of calcium orthophosphates. *Biomaterials*. 31: 1465-1485

9. Einhorn T. 2001. The cell and molecular biology of fracture healing. *Folia Traumatologica Lovaniensia*. 86-103

10. Willerth SM, Arendas K, Gottlieb D, Sakiyama-Elbert S. (2006) Optimization of fibrin scaffolds for differentiation of murine embryonic stem cells into neural lineage cells. *Biomaterials* 27: 5990-6003

11. Zhao H, Ma L, Gao C, Wang J, Shen J. (2009) Fabrication and properties of injectable β-tricalcium phosphate particles/fibrin gel composite scaffolds for bone tissue engineering. *Materials Science and Engineering* 29: 836-842

12. Toquet J, Rohanizadeh R, Guicheux J, Couillaud S, Passuti N, Daculsi G, Heymann D. (1999) Osteogenic potential in vitro of human bone marrow cells cultured on macroporous biphasic calcium phosphate ceramic. *J Biomedical Materials Research.* 44: 98-108

13. Le Nihouannen Daclusi G, Saffarzadeh A, Gauthier O, Delpace S, Pilet P et al. (2005) Ectopic bone formation by microporous calcium phosphate ceramic particles in sheep muscles. *Bone*. 36: 1086-1093

14. Palmer L, Newcomb C, Kaltz S, Spoerke E, Stupp S. (2008) Biomimetic systems for hydroxyapatite mineralization inspired by bone and enamel. *Chem Rev.* 108: 4754-478

15. De Bruijn J, Van Blitterswijk C, Davies J, (1995) Initital bone matrix formation at the hydroxyapatite interface in vitro. *J Biomedical Material Research* 29:89-99

16. Kim SB, Kim YJ, Yoon TL, Park S, Cho IH, Kim EJ, Kim IA, Shin J. (2004) The characteristics of a hydroxyapatite-chitosan-PMMA bone cement. *Biomaterials* 25: 5715-5723

17. Deligianni D, Katsala N, Koutsoukos P, Missirlis Y. (2000) Effect of surface roughness of hydroxyapatite on human bone marrow adhesion, proliferentiation, differentiation, and detachment strength. *Biomaterials* 22: 87-96

18. Bensaïd W, Triffitt J.T, Blanchat C, Oudina K, Sedel L, Petite H. (2003) A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. *Biomaterials.* 24:2497-2502

19. Cordonnier T, Layrolle P, Gaillard J, Langonné A, Sensebé L, Rosset P, Sohier J (2009) 3D envoronment oh human mesenchymal stem cells differentiation for bone tissue engineering. *J Material Science*. 21: 981-987

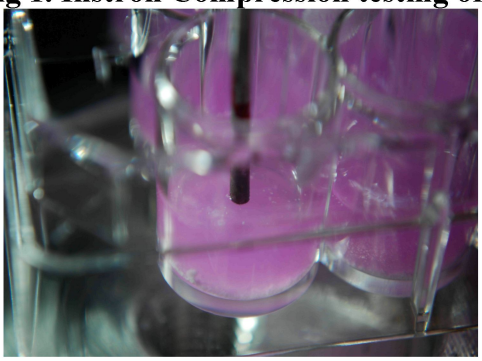
20. Salinas C.N, Anseth K.S. (2009) Mesenchymal stem cells for craniofacial tissue regeneration: designing hydrogel delivery vehicles. *J Dental Research* 88: 68

Appendix

Table 1. AlamarBlue® redox indicator selected by AbD Serotec for assessment of metabolic proliferation without interruption of the electron transport chain

Half-reaction			Eo' (mV) pH7.0 25°C
$NAD+2H^++2e^-$	<----->	$NADH+H^+$	-320
$NADP+2H^++2e^-$	<----->	$NADPH+H^+$	-320
$FAD+2H^++2e^-$	<----->	$FADH_2$	-220
$FMN+2H^++2e^-$	<----->	$FMNH_2$	-210
$MTT_{OX,} + 2H^+ + 2e^-$	<----->	MTT_{RED}	-110
$cytochromes_{OX,}+1e^-$	<----->	$cytochromes_{RED}$	+80 to +290
$alamarBlue^{®}{}_{OX}+2H+2e^-$	<----->	$alamarBlue^{®}{}_{RED}$	380
$O_2+4H^++4e^-$	<----->	$2H_20$	820

Fig 1. Instron Compression testing of an HA-TCP construct



Vita

Roxana Fard completed her Bachelor of Science at California Lutheran University in 2005. Following the completion of her degree she went to work as an industrial microbiologist for Meissner Filtration Products, Inc. She entered into the Masters program at California State University Channel Islands in 2008. In 2009 she was one of 14 students selected to partake in a stem cell research internship funded by the California Institute for Regenerative Medicine and Extended Education at CSUCI. Her research project has been under the mentorship of Dr. Bill Tawil at the University of California Los Angeles in the Department of Material Science and Engineering.