The Influence of Peptide Modifications of Bioactive Glass on Human Mesenchymal Stem Cell Growth and Function

Mohamed Ammar
Lehigh University
The Influence of Peptide Modifications of Bioactive Glass on Human Mesenchymal Stem Cell Growth and Function

By

Mohamed Ammar

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Date

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Thesis Advisor

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Chairperson of Department
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Abstract

Bioactive glass is known for its potential as a bone scaffold due to its ability to stimulate osteogenesis and induce bone formation. Broadening this potential to include the differentiation of human mesenchymal stem cells (hMSCs) to bone cells will enhance the healing process in bone defects. The surface of bioactive glass made by the sol-gel technique with the composition of 70% SiO$_2$-30% CaO (mol %) was grafted with 3 peptides sequences in different combinations from proteins (fibronectin BMP-2 and BMP-9) that are known to promote the adhesion, differentiation and osteogenesis process. The experiment was done in two forms, a 2D non-porous thin film and a 3D nano-macroporous structure. hMSCs were grown on the materials for a total of five weeks. The 2D materials were tested for the expression of 3 osteogenic markers (osteopontin, osteocalcin and osteonectin) through immunocytochemistry. The 3D forms were monitored for cell’s adhesion, morphology, spreading and proliferation by scanning electron microscopy, in addition to proliferation assay and alkaline phosphatase activity measurement. Results showed that hMSCs poorly adhered to the 2D thin films, but the few cells survived showed enhanced expression of the osteogenic markers. On the 3D form, cells showed enhanced proliferation at week one and more survival of the cells on the materials grafted with the adhesion peptide for the successive weeks in comparison to the positive control samples. Enhanced alkaline phosphatase activity was also detected compared to the negative control samples but were still below the positive control samples. In conclusion, the peptide grafting could increase the effect of bioactive glass
but more peptide combinations should be examined to improve the effects on the differentiation and osteogenic activity of the hMSCs.
Chapter 1: Background

1.1. Problem History:

Bone is a remarkable tissue playing key roles in critical functions in human physiology including protection, movement and support of other critical organs, blood production, mineral storage and homeostasis, blood pH regulation, multiple progenitor cell (mesenchymal, hemopoietic) housing, and others (Porter et al. 2009). As age advances, bone density decreases and the strength of bone is decreased as a result. These effects are particularly apparent after the age of 30, and are typically more severe in women due to the hormonal changes associated with menopause. Bone making cells lose their productive efficiency to make new bone and repair fractures. Consequently, many people of advanced age suffer from collapsed vertebrae and broken hips (Hench 1998).

Skeletal defects and fractures due to trauma, tumor removal, and age-related diseases such as osteoporosis and osteoarthritis are frequently seen in the dental and medical fields. Their repair in most of the cases is a major problem with its results having great impact on the health and quality of life of the population. Bone problems account for more than one million patients every year (Haralson & Zuckerman 2009). Most of these patients are treated with osteosynthesis material such as hydroxyapatite and tricalcium phosphate graft materials and approximately 80% of these require adjuvant grafting. Many require tissue replacement with ‘bioinert’ materials, such as metal alloy hip replacements (Ducheyne & Qiu 1999).
In 2004, musculoskeletal conditions imposed an $849 billion burden on the United States economy alone, which equates to 7.7% of the national gross domestic product. The financial burden of musculoskeletal conditions is expected to escalate in the next 10-20 years due to the aging population, and the increased sedentary lifestyle of many individuals. More research to find better treatment solutions or enhance the current ones is urgently required if health and economic burden to be contained or lowered (“The Burden of Musculoskeletal Diseases in the United States”, 2008).

Bone is a unique tissue in that it can heal itself without scarring, a process referred to as regeneration. However in humans, this process is limited by the defect size. As humans, unlike species such as salamanders and geckos, have a limited ability to completely regenerate large tissue defects (Tanaka 2003). In large defects, cells need a substrate on which to adhere and fill the defect. If an appropriate substrate is not available, the repair or regeneration process will not be complete. To overcome this limitation, surgeons have used different graft materials to help aid the body repair the defects (Jones 2009).

The graft materials used for this purpose are classified in several categories, including: autogenic, allogenic, xenogenic or alloplastic. The gold standard in reconstructive surgery for damaged or diseased bone is the autogenic graft, which involves transplantation of the patient’s own tissue from a donor site to the damaged site. Another option is the allogenic graft, which involves tissue transplantation from another patient. In contrast, xenogenic grafts are tissues harvested from a different species, such
as freeze-dried bovine bone that are transplanted into patients at the site of the defect. Finally, alloplastic solutions are synthetic grafting materials (Murugan & Ramakrishna 2007).

Unfortunately, each technique is limited. Autografts create a second surgical site from which the patient must recover. The additional recovery time can extend a patient’s hospital stay and subsequently the hospitalization expenses. This secondary site could be uncomfortable for years after the surgery and death of its healthy tissue may occur. In addition, it is difficult to find the suitable autograft for the fracture site to be repaired in regard to size, shape and bone quality (Chen et al. 2005). Allografts take a longer time (compared to autograft tissue) to be incorporated into the recipient’s body, carry the risk of disease transmission and are in short supply. Xenografts are in large supply but they have significant risks of immune rejection, in situ degeneration and disease transmission. Alloplastic implants experience a limited lifetime in clinical use, which can be attributed to material properties that limit long term success. These deficiencies of alloplastic grafts over natural tissues include: the ability to self-repair, the ability to maintain a blood supply and the ability to modify in response to stimuli such as mechanical load (Jones et al. 2007).

These complications associated with these types of graft materials demonstrate the need for alternative options. Materials that are capable of facilitating functional bone formation, stimulate the body’s own regenerative mechanisms, restore diseased or damaged tissue to its original state and function, need to be developed (Ingber et al.)
2006). For that regenerative medicine strategies and tissue engineering are currently in development.

1.2. **Tissue engineering as an alternative:**

Tissue engineering and regenerative medicines are promising new scientific fields that exhibit significant clinical promise. Tissue engineering is defined as “an interdisciplinary field in which the principles of engineering and life sciences are applied toward the generation of biologic substitutes aimed at the creation, preservation or restoration of lost organ function” (Vacanti & Vacanti, 2000). It is the science of regenerating the human tissues where a loss or damage has occurred due to injury or disease in which the normal healing process of the body will not be able to restore this damage completely. The concept is based on the use of a template (scaffold) tailored with specific characteristics that mimic the extra cellular matrix (ECM) of the application site tissues, incorporating stimulating molecules and/or cells for the tissue to heal and restore its function. In some cases, the scaffold alone is sufficient to use in the defect (Singh et al. 2008).

![Figure 1.1: Basic tissue engineering principles and steps.](image-url)
Ideally, a scaffold should mimic the structure and biological function of the ECM both in terms of chemical composition and physical structure. For example in a typical connective tissue, structural protein fibers such as collagen and elastin entangle with each other to form a non-woven mesh that provides specific tensile strength and elasticity for every type of tissue. It also provides a substrate with specific ligands for cell adhesion and migration, and regulates cellular proliferation and function by providing various growth factors (Ma et al. 2005).

1.3. Bone Scaffold Requirements:

There are several criteria that are required to be present in a bone scaffold for successful tissue regeneration. The material should be non-toxic, incite only a minimal inflammation response and not cause scar tissue formation around it, allowing for direct bond with the bone tissue in the site of application. The material should possess mechanical strength that is equivalent to the implantation site. This will prevent stress shielding, allowing for native tissue and implant integrity. It should consist of a highly interconnected porous network to allow for tissue ingrowth, new vascularization formation, nutrition delivery and waste removal. The scaffold should dissolve or degrade at an equivalent rate at which new tissue forms, so that the tissue

Figure 1.2: Computer model of proposed bone scaffold (Jones 2007)
replaces the scaffold as it grows. Meanwhile, the scaffold should maintain sufficient strength as it degrades to support the forming tissue until it gain its own strength. The degradation product should be nontoxic and easily excreted from the body by its own mechanisms (Jones 2009, Murugan & Ramakrishna 2007). In addition to these above basic criteria, it is beneficial for better and faster outcome that the scaffold material possess an inductive effect on the cells, meaning to be able to induce faster bone formation through providing chemical and/or physical means to the cells to enhance its differentiation and productivity to produce the bone matrix. This inductive effect helps faster the healing process which in turn will decrease the patient discomfort and hospitalization period (Porter et al. 2009).

The incorporation of bioactive signaling molecules in/on the material of the scaffold that the cells receptor can interact with and develop a specific desired responses by the cells, may greatly enhance the performance of the scaffold. These molecules can play a crucial role in developmental and biological repair processes, including morphogenesis, wound healing, the immune response, and angiogenesis, where cellular migration and/or differentiation is sensitively governed by the spatial distribution of these chemical signals. The best way to choose the right chemical signals to be added is by looking at the native ECM of bone and see what proteins are making up its structure. Growth factors such as platelet-derived growth factors (PDGFs), bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), and transforming growth factor-βs (TGF-βs) are found in the bone ECM and have an indisputable role in osteoinduction and osteoconduction (Singh et al. 2008).
Cells interact with the ECM proteins through a ligand-receptor binding interaction mediated by transmembrane proteins and specific domains on the proteins of the ECM. This cell–matrix interaction produces specific cell responses that influence cell adhesion, motility, shape, orientation, differentiation, and survival (Singh et al. 2008). Incorporation of short peptides sequences instead of the whole protein molecule that represent domains that are known to affect the bone cells adhesion, differentiation and function, in the scaffold materials, would give control over the cell responses and increase the potential of the bone scaffold. The RGD sequence, for example, is found in active fibrinogen, laminin and fibronectin proteins (Ruoslahti & Pierschbacher, 1987), that mediate cell adhesion, while YIGSR and IKVAV are both found in laminin, that also play a key role in cell attachment, and migration (Yamada 1991, Zamora et al. 1993). Each of these domains is known to influence cell adhesion when incorporated into a solid surface (Dettin et al. 2006). Other specific peptide sequences from the bone ECM proteins and growth factors, like BMPs and TGF-βs, can be incorporated that can play crucial role in regulating the cellular physiology leading to enhanced bone matrix formation in the scaffold.

Cells can also sense physical characteristics of the scaffolds, such as: wettability, roughness, crystallinity, and surface charge. These may critically influence the host response to the engineered construct by affecting protein–biomaterial and cell–biomaterial interactions in vitro or in vivo, directly or indirectly (Singh et al. 2008). Composition, topography and architecture of scaffolds are able to interact and influence cell behavior, through triggering different intracellular reactions that are mediated by the
adhesion receptors, able of controlling gene expression. Scaffold architecture has been shown to modify the response of cells and subsequent tissue formation, as seen by the mineralization of specific areas in the body of scaffolds (Ripamonti 2004). Nano to microscale topography has been demonstrated to affect cell behavior by modification of cytoskeleton arrangements as different scale topography possess range of surface chemistry and energy, that directly affect the interaction of the cell receptors and the adsorption of the proteins on its surfaces. Accordingly cell receptors mediated signals affect the cytoskeleton arrangement (Figure 1.3) which can be used in the engineered bone scaffold to guide the cellular organization (Wilkinson et al. 2002).

Figure 1.3: Epitenon (tendon) cells on and off a grooved substrate. Grooves 7 mm wide, 3 mm deep and spaced by 14 mm. (Wilkinson et al. 2002)
1.4. Materials For Bone Regeneration:

A variety of scaffold materials and fabrication techniques has been developed in trials to make a material that mimic the ECM, including: ceramics, synthetic and natural polymers, metals, and composites of them. Among these materials, ceramics are the most used materials for bone regeneration due to similarity of the compositions used to the composition of bone and their ability to support bone cells function. However often unsatisfactory effectiveness in clinical repair is found because of the mismatch as in mechanical compatibility with surrounding tissue, limited porosity, inflammatory response, mechanical instability during loading, and rates of degradation (Yang et al. 2001).

Four decades ago, certain silicate-based glass compositions were found to bond chemically to bone. These glasses were later called bioactive glasses because of the surface reaction they elicit on their surface when exposed to a physiologic environment that lead to the formation of a hydroxyl apatite layer. This layer bond to collagen produced by the bone cells and subsequently to bone matrix, forming strong bond to bone which is a very important property for the success of any bone filling or regenerating material (Hench 2006, Best et al. 2008).

Another important property for these glasses is that they leach ions in certain concentrations upon their dissolution in the physiologic environment. These ions up-regulate the expression of the genes responsible for osteogenic differentiation in the pre-osteoblastic progenitor (Xynos et al. 2001) as while as inducing the release of angiogenic
growth factors that lead to the formation of new vascularization (Day 2005). This later property gives advantage for these bioactive glasses over the other biomaterials for better integration with the tissues.

1.5. Cells For Bone Engineering:

The appropriate use of specific cells is one of the defining factors in the success of tissue engineering. Many studies have been conducted to identify the right types of cells for the construction or regeneration of each tissue/organ. Although the specialized cells remain an important source for tissue engineering, the use of stem cells has recently been recognized as a promising alternative to specialized cells owing to their enormous potential in generating a spectrum of tissues with adequate functions (Murugan & Ramakrishna 2007).

Mesenchymal stem cells (MSCs) are pluripotent cells capable of differentiation into different cell phenotypes. One of these phenotypes is bone cells (osteoblasts) which can easily be induced in vitro by the use special culturing media that contain chemicals such as dexamethasone, ascorbic acid, and β-glycerol phosphate (Pittenger et al. 1999). MSCs differentiated towards bone forming cells, are able to produce a bone matrix on a synthetic substrate ex vivo. Although some studies have shown enhanced osteogenic capacity and better integration with the bone of the implantation site when compared to transplanted scaffolds alone (Mauney et al. 2005), other number of studies have shown
that MSCs which have been cultured *in vitro* over repeated passages lose their osteodifferentiation and bone forming capacity once implanted *in vivo* (Banfi et al. 2000).

Other obstacles confronted in this approach for translating of this technology from the bench to the bedside involves the additional surgery that is needed to obtain the MSCs from the patient, and the waiting period the patient has to wait for the graft to develop *in vitro*. As, they are present in relatively low concentration in the bone marrow as compared to other cell types and their doubling time is relatively slow making it difficult to obtain sufficient cell density in a large scaffold. Moreover sterilization techniques need to be established for the cell-scaffold construct that had been prepared *in vitro* (Porter et al. 2009). Therefore, there is a need for novel culturing techniques to promote the rapid development of a transplant-ready cellular scaffold through accelerating the cellular production of ECM and preservation of the cells phenotype. On that bases our objectives in this study was set up to create a substrate that can support and maintain the cells adhesion, growth, and differentiation to bone cells for bone cells. In addition to enhancing the bone cells function for better integration in the application site and faster healing through enhanced bone tissue regeneration.
1.6. Objectives of the study:

The main goal of this study is to develop a scaffold that can support the adhesion, and proliferation of hMSCs, direct their differentiation toward the osteogenic phenotype and enhance the function of the differentiated bone cells for better bone regeneration. Therefore our objectives in this study summarizes in: 1) the preparation of peptide sequences from proteins known for their role in bone formation to be used as chemical cues in the scaffold. 2) Culturing human mesenchymal stem cells and investigating their differentiation potentials. 3) Preparation of 2D thin films and 3D porous scaffolds of bioactive glass material with the incorporation of the prepared peptides into their structure. 4) Investigating the potential of the prepared scaffolds on supporting the adhesion, differentiation and osteogenic performance of the hMSCs.

Figure 1.4: Illustrative diagram for the stem cells isolation, culture, seeding and implantation. (Porter et al. 2009)
Chapter 2: Literature Review

2.1. Bioactive materials for Bone Regeneration:

2.1.1. Background:

Ceramic materials are widely utilized in the medical field for variety of purposes, such as eye glasses, diagnostic tools, fiber optics for endoscopy, dental restoration, and many others. Half a century ago, certain compositions of ceramic materials started to be used for the reconstruction and repair of bone defects in the human body. These ceramics were coined bioceramics. These ceramics demonstrated good biocompatibility, osteoconductivity and biodegradability of some of them (Hench 1998, Oh 2006).

Bioceramics can be polycrystalline hydroxyapatite, bioactive glasses, or bioactive glass-ceramic (Hench 1998). Glass-ceramics are partially crystallized glasses through controlled heat treatment that contain participated crystals like apatite and wollastonite (e.g. A/W glass ceramics). Bioceramics may further be subcategorized as either “bioinert” or “bioactive”, where the bioinert ceramics are biologically non-active and do not bond to tissues. Bioactive ceramics induce specific responses in the cells along the material-tissue interface that lead to bonding between them. The bioactive ceramics also may be resorbable or non-resorbable (Best 2008). Each subcategory varies with respect to the activity and performance resulting from small changes in the structure and composition of the material.

Hydroxyapatite (HA), tricalcium phosphate (TCP) and bioactive glass (BG) are the three top used bioceramic materials due to their direct bond to the surrounding osseous
tissue and their effect in enhancing the process of new bone tissue formation (Hench 1998, Ducheyne & Qiu 1999). Although their successful use in bone defects, every one of them has some drawbacks. Stoichiometric HA (exact whole-number ratios of compositional elements are present) has the disadvantage of low rate of bone bonding which has implications for the time required for patient recovery, in addition to a very slow degradation rate in contrast to TCP and BG. TCP on the other hand, has a high rate of degradation which is not always favorable in some cases such as big defects where the rate of bone formation is slower than the rate of the material resorption. BG has the disadvantage of weak mechanical properties. Specifically, the sol-gel derivatives exhibit severely limited mechanical strength, which limits its use in load bearing defects. This disadvantage is outweighed by the fact that BG has the highest bioactivity among the bioceramic materials. The bioactivity of BG has led to an increased interest in the field of tissue engineering and regenerative medicine (Rahaman et al. 2011).

2.1.2. Bioactive glasses:

Bioactive glasses have been found to bond more rapidly to bone and have a rate of degradation which is between the HA and TCP. Bioactive glasses also have the property of being osteoinductive, meaning they stimulate new bone growth on the implant away from the bone–implant interface. In contrast to the osteoconductive materials e.g. HA that encourages bone to grow only along the implant at the bone–implant interface (Oonishi et al. 1999).
The osteoinduction property of the bioactive glasses is attributed to the release of its dissolved elemental constituent such as silicon (as silicic acid), calcium, sodium and phosphate species as it degrade into the physiological environment. It is thought that a combination of some of these ions (such as silicon and calcium) triggers the osteoprogenitor cells to differentiate and produce new bone (Hench & Polak, 2002). Molecular biology studies have shown that seven families of genes involved in osteogenesis are stimulated by bioactive glass dissolution products, including insulin growth factor II (IGF-II), IGF binding proteins, and various proteases that cleave IGF-II from their binding proteins (Xynos et al. 2001). IGFs are involved in the synthesis of collagen by osteoblasts, an essential component of bone. It is accepted that the rate and type of dissolution ions released from bioactive glasses determine gene expression (Figure 2.1), however, the intracellular signaling pathways remain uncertain (Jell et al. 2006).

Figure 2.1: Gene expression regulation mechanisms by bioactive glasses (Jell et al. 2006).
The bioactive glasses bond to bone through the formation of hydroxycarbonate (HCA) layer on its surface when placed in the body fluids. A series of chemical reactions happen on the surface when it is exposed to the body fluids that lead to the formation of this layer (Pantano et al. 1974, Hench 1998). Five reaction stages (Hench & West, 1996) lead to the formation of a strong bond between bioactive glass and the tissues at the implantation site. These stages involve the rapid release of soluble ionic species through glass corrosion or dissolution, which lead to the formation of a bilayer composed of high-surface-area hydrated silica layer and polycrystalline HCA layer on top of the former (Jones 2007). The specific stages are described below:

**Stage 1:** Rapid exchange of Na+ or Ca2+ with H+ or H2O+ from solution, causing hydrolysis of the silica groups, which creates silanol (Si–OH) groups: e.g. Si–O–Na+ + H+ → Si–OH + Na+(aq)

**Stage 2:** Soluble silica is lost in the form of Si(OH)4 to the solution, resulting from the breaking of Si–O–Si bonds and the continued formation of Si–OH (silanols) at the glass–solution interface

**Stage 3:** Condensation and repolymerisation of the Si–OH groups is then thought to occur, leaving a silica-rich layer on the surface, depleted in alkalis and alkali-earth cations.

**Stage 4:** Ca2+ and PO43− groups then migrate to the surface through the silica-rich layer and from the surrounding fluid, forming a CaO–P2O5-rich film on top of the silica-rich layer.

**Stage 5:** The CaO–P2O5 film crystallizes as it incorporates OH- and CO3 2− anions from solution to form a mixed HCA layer.

Figure 2.2: The surface reaction stages of bioactive glass.
The biological mechanisms of bonding that follow HCA layer formation are thought to involve the adsorption of ECM proteins and growth factors, followed by the attachment, proliferation and differentiation of osteoprogenitor cells to mature bone forming cells (Hench & Polak, 2002). Osteoblasts (bone-forming cells) lay down the bone extracellular matrix (collagen matrix), which then mineralizes to create a nanocomposite of mineral and collagen on the surface of the bioactive glass implant while the dissolution of the glass continues over time (Ducheyne & Qiu, 1999). The rate of these surface interactions is affected by the composition and structure of the bioactive glasses. Bioactive glasses can be prepared through either one of two techniques that lead to different structures and compositions.

Figure 2.3: The events sequences that occur on the glass implant surface in a physiological environment (Ducheyene & Qiu, 1999).

[(1) ions dissolution; (2) reprecipitation from solution; (3) ion exchange and structural rearrangement at the ceramic-tissue interface; (4) interdiffusion from the surface boundary layer into the ceramic; (5) solution-mediated elects on cellular activity; (6) deposition of either the mineral phase (a), or the organic phase (b), without integration into the ceramic surface; (7) deposition with integration into the ceramic; (8) chemotaxis to the ceramic surface; (9) cell attachment and proliferation; (10) cell differentiation; and (11) extracellular matrix formation]
2.1.3. Bioactive glasses preparation techniques:

2.1.3.1. Melt quench technique:

Traditionally, glasses and ceramics are prepared by mixing various metal oxides, nitrates, and other compounds together, followed by heating them to a high temperature (~1300-2000°C) until they melt. This process requires a significant amount of energy and results in the transformation from a highly ordered crystalline precursor state, to a covalently bonded amorphous, random network state. These glasses are known as melt-derived glasses. The first bioactive melt-derived glass was the Bioglass® 45S5 (the 45 representing 45 wt% SiO₂, S being the network former and 5 representing the ratio of CaO to P₂O₅) originally reported by Hench in the early 70s (Hench et al. 1971).

![Figure 2.4](image_url): Temperature versus time graph showing required temperatures and times for processing of different types of bioactive glasses (Hench 1998).
The bioactivity of the melt-derived bioactive glasses and glass ceramics depend on the rate of HCA layer formation, which changes with the elemental type and concentration making up the composition. The bioactive glasses with the highest level of bioactivity lie in the middle (region S) of the Na$_2$O–CaO–SiO$_2$ diagram (Figure 2.5) (assuming constant 6 wt% P$_2$O$_5$) (Hench 1998). In region S, bioactive glasses not only bond to bone but also are osteoinductive. Compositions that exhibit slower rates of bonding lie between 52 and 60 wt% SiO$_2$ in the glass. Compositions with greater than 60 wt% SiO$_2$ (region B) are bioinert (Jones et al. 2007), in contrast to bioactive glasses prepared by the sol-gel technique, whose compositions with up to 80% mol SiO$_2$ retain their bioactivity.

Figure 2.5: Composition diagram for the bioactivity of melt derived silicate glass (Jones et al. 2007)
2.1.3.2. Sol-gel technique:

One important development in materials synthesis and processing is that of the sol-gel process. Sol-gel is essentially a low temperature, chemical route to the production of glasses and glass-like materials. This alternative approach is known for more than a century, but it has gained a new importance in the last three decades (Sepulveda et al. 2002). The sol-gel process takes an organo-functionalized metal or metalloid center, and reacts that in an aqueous environment to form a covalently bonded random network material, so that duplicating the high temperature glass-making process in that the final form is an amorphous solid (Abiraman et al 2002, Saravanapavan et al 2003).

In the case of the bioactive glasses, the organo-functionalized metal is silica, often in the form of tetramethyl orthosilicate or tetraethyl orthosilicate. When this compound is added to water, under acidic conditions, hydrolysis occurs, which forms a sol. The sol can be considered as a solution of silica species that can undergo polycondensation to form the silica network of Si–O–Si bonds (Hench & West, 1990) and then a gel forms as the silica network grows. If components other than silica (Ca, etc.) are required in the glass composition, they are added to the sol either as other alkoxides or as salts. Water and ethanol are produced as by-products of the condensation reaction. These are later removed by evaporation under carefully controlled low heating rates during the drying process. The final step is to heat the dried gel to at least 600°C in order to remove organic by-products and stabilize the composition (Saravanapavan & Hench, 2003).
2.1.4. Advantages of sol-gel over melt derived glasses:

Sol–gel glasses have enhanced resorbability, bioactivity and bone bonding capabilities \textit{in vivo}. A comparison study for \textit{in vivo} resorption of 45S5 glass particles and bioactive gel glass particles (58S and 77S) with starting average size of the 45S5 particles smaller than the 58S and 77S particles, showed that within 12 weeks of implantation in rabbits, the gel-glass particles had significantly resorbed when compared with the 45S5 particles. In addition, there were significantly more 45S5 particles remaining in the defect after the 12 weeks (Wheeler et al. 2000). Molecular control over the texture and rate of dissolution of bioactive sol-gel glasses shows that it is possible to produce a material that resorbs at the same rate as new trabecular bone is formed and give regenerated bone with similar mechanical properties to normal host bone (Hench et al. 1998).

Sol–gel derived bioactive glasses are generally more bioactive, and can remain bioactive with silica contents of up to 80 mol\% in contrast to melt-derived glasses that loss their bioactivity above 60 mol\%. The enhanced bioactivity is due to gel glasses contain a nanoporous network that is inherent to the sol–gel process, whereas melt-derived glasses are fully dense. That causes increased rates of dissolution, accelerating the exchange of Na\(^+\) or Ca\(^{2+}\) with H\(^+\) or H\(_3\)O\(^+\) from solution and causing rapid hydrolysis of the silica groups (Jones et al. 2007).

Sol–gel glasses have a specific surface area about two orders of magnitude greater than that of melt-derived glasses due to this nanoporous network, typically ~200 m\(^2\) g\(^{-1}\). The nanopores in sol–gel glasses are usually in the range of 1–30 nm in diameter. This
size can be tailored during processing by controlling the pH of the catalyst, the nominal composition and the final temperature (Saravanapavan & Hench, 2003). A large surface area rich in silanols make it possible for the bioactive gel-glasses to nucleate a biologically active HCA layer within minutes, even more rapidly than do bioactive melt glasses with much lower silica contents (Hench 1998).

Sol gel glasses can also be bioactive while containing fewer components, e.g. glasses composed of 70 mol% SiO₂ and 30 mol% CaO (70S30C) form an HCA layer as rapidly as the 60 mol% SiO₂, 36 mol% CaO and 4 mol% P₂O₅ (58S) glass (Saravanapavan et al. 2003). The reason that 70S30C glass can nucleate an HCA layer even though it does not contain phosphate is that Si–OH groups are thought to play a role in HCA layer nucleation. These groups form during the bioactivity mechanism (glass corrosion), but in sol–gel glasses there are several Si–OH groups present in the unreacted glass that can quickly act as nucleation sites. HCA layers have been shown to nucleate on various materials (e.g. polymers) that have a high concentration of surface OH groups when the materials are placed in supersaturated solutions (Li et al. 1994, Miyazaki et al. 2003).

The surface of sol–gel glasses can be modified by a variety of surface-chemistry methods, e.g. with amine groups, and be attractive to specific proteins such as laminin. Specially designed proteins for inducing specific cellular responses can also be attached to the material surface prior to implantation to obtain novel bioactivity by delivering the proteins to the desired wound site (Lenza et al. 2003). These modifications can be used to
design materials that can provide specific stimuli to cells leading to the ability to guide desired tissue regeneration in the essence of the tissue type, organization and 3D structure with the later being tailored through the scaffold design features such as size, shape, porosity and interconnectivity.

2.1.5. Porous Sol-Gel bioactive glasses as bone scaffolds:

The sol gel technique offer feasibility for tailoring the shape of the glass while it is still in the sol state. Many attempts has been done to produce macro-porous bioactive glass scaffolds including the use of foaming agents (hydrogen peroxide, surfactants) and porogenic agents (polystyrene spheres, polyvinyl alcohol, polyethylene glycol, etc.) (Yuan et al. 2001, Yan et al. 2001, Jie et al. 2004, Li et al. 2005). Sepulveda and co-workers developed a foaming process to produce macroporous gel-glasses (Sepulveda et al. 2002). This process was based upon the gel-casting technique previously developed by Sepulveda to produce macroporous ceramics (Sepulveda & Binner, 1999). The concept was applied to sol-gel processing by foaming small quantities of the sol using vigorous mechanical agitation, a surfactant and a gelling agent. The glass foams produced exhibited a hierarchical structure with interconnected macropores (10–500 μm) and a mesoporous framework (pores of 2–50 nm), thus fulfilling the pore size criterion for tissue engineering scaffolds. Jones et al utilized X-ray microcomputed tomography (μCT) to study the porosity of glass foam scaffold. The images obtained showed that the macropores are well interconnected. In fact, the pore structure is hierarchical because the nanoporosity inherent to the sol–gel process is maintained (Jones et al. 2007). This
nanoporosity is beneficial to cells as it mimics the hierarchical structure of natural tissues and, therefore, more closely simulates a physiological environment that stimulates cell behavior than a surface without nanopores. Compressive strength of this foam scaffold with 100 µm diameter pores was 2.4 MPa, which is similar to those of porous bone and clinically used porous hydroxylapatite (Valentini et al. 2000).

Polymers also have been used as pore formers to introduce additional macropores. Nanoporous (2–10 nm) and macroporous (0.1–40 µm) samples have been fabricated on the basis of polymerization-induced phase separation, in various silica-based sol-gel systems, by using organic polymers such as poly(ethylene oxide) (PEO), poly(ethylene glycol) (PEG) or poly(acrylic acid) (HPAA) (Nakanishi 1997, Takahashi et al. 2005). In 2007, Marques et al. reported fabrication of sol-gel bioactive glass with bimodal porosity in 70% SiO₂–30% CaO and 77% SiO₂–19% CaO–4% P₂O₅ glass compositions using the polymerization-induced phase separation technique. The macropores were in the range of 100 um and the nanopores sizes were increased by using solvent exchange to 18 nm (Marques et al. 2007).

The compressive strength of porous bioactive glass scaffolds is still not suitable for load-bearing defect sites and in sites where there is dynamic high-load environment such as the hip. One of the ways to produce a porous scaffold with the bone-bonding and cell-stimulating properties of a bioactive glass with higher strength and toughness is to create an inorganic–organic nanocomposite by incorporating biodegradable polymers into the sol–gel process (Pereira et al. 2005; Vallet-Regí et al. 2006). A scaffold with this
composition would mimic the structure of natural bone, which is a composite of brittle hydroxyapatite and tough collagen (Jones 2010).

2.2. Mesenchymal Stem Cells:

2.2.1. Introduction:

Multicellular organisms have the ability to regenerate and heal their tissues through the availability of unspecialized cells within their tissues. These cells are called stem cells. They are characterized by having high capacity for self renewal and the ability to give rise to progeny that are committed to differentiation into diverse specialized cell types. There are two broad types of stem cells: embryonic stem cells that are isolated from the inner cell mass of blastocysts, and adult stem cells that are found in various tissues (Shenaq et al. 2010). The stem cells derived from embryonic tissues can differentiate and renew almost any tissue, whereas adult stem cells have limited capacity usually restricted to cell types of the tissue that they originally came from (e.g., hematopoietic stem cells, neural stem cells, epidermal stem cells, mesenchymal stem cells (MSCs)) (Choumerianou et al. 2008).

The regenerative potential of stem cells lends itself to finding a renewable source. One source that researchers have identified is cells in the bone marrow. For decades, it has been known that the bone marrow contains two types of stem cells: hematopoietic ones, which are committed to differentiate into mature blood cells, and the less-differentiated stromal mesenchymal cells. The MSCs are non-hematopoetic, stromal cells that exhibit multilineage differentiation capacity, being capable to give rise to different
tissues. They are composed of heterogeneous population of plastic-adherent, fibroblast-like cells (Bourin et al. 2008).

### 2.2.2. History of MSCs:

In the 1960s, Alexander Friedenstein, was the first researcher to discover a multipotent progenitors cells isolated from bone marrow. He stated the critical observation that bone marrow in postnatal life is a reservoir of stem cells for mesenchymal tissues. For the first time, from rodent bone marrow, he isolated and cultivated, cells that formed colonies of long adherent, fibroblast-like cells. These colonies came from only one cell type, named colony-forming unit-fibroblasts (CFU-Fs) and they were able to differentiate to bone and hematopoetic cells (Afanasyev et al 2009).

In the early 1990s, Arnold Caplan named CFU-Fs mesenchymal stem cells (MSC) and showed that they can also differentiate to cartilage, tendons, and muscle and not only bone and medullar stroma (Caplan 1991). They are also referred to as marrow stromal cells. In 2005, the MSCs were defined as multi-potent mesenchymal stromal cell by the International Society for Cellular Therapy, due to the lack of evidence that these cells have all properties of stem cells (Bourin et al. 2008). Although of the different acronyms, they are all abbreviated to MSCs.

### 2.2.3. MSCs Function:

MSCs act as replacement units for the specialized cells that naturally expire or die due to injury or disease (Caplan 2007). They have the ability to differentiate in vivo and
in vitro into a variety of adult mesenchymal tissues, such as bone, cartilage, adipose, and muscle. They are characterized by the secretion of wide range of bioactive molecules that regulate immune responses and create a suitable environment for regeneration at the sites of tissue injury. Therefore they are considered to be an important counterpart for potential clinical applications (Caplan 2007, Schafer & Northoff 2008, Bieback 2008).

2.2.4. Sources of MSCs:

Almost all tissue types in the body have an endogenous population of MSCs. The MSCs can be found in bone marrow, adipose tissue, muscles, cartilage, synovium, synovial fluid, tendons, placenta, umbilical blood, periostium, spleen, thymus, connective tissues and teeth pulps (Schäfer et al. 2008). Of these, adipose tissue and bone marrow offer advantages in terms of access, abundance, and the extent of their documentation in the literature. MSCs seem to reside in prevascular niches as hypothesized from their distribution throughout the body (da Silva Meirelles et al. 2006). It is assumed that they are present in very low numbers in the peripheral blood of adult humans in the absence of pathological status (Schäfer et al. 2008). Although they were not isolated from adult human peripheral blood, there are reports on isolation of MSCs from the peripheral blood of rats and pigs (Faast et al. 2006).

MSCs can also be isolated from the blood of the umbilical cord. The possibility of obtaining MSCs from Cord Blood (CB) is approximately 60% at the maximum. The critical parameters for success are the time between harvest and the beginning of culture (15 h seems to be the maximum), the volume of CB (>33 ml), and the total quantity of
mononuclear cells. After cryopreservation, the chances of success are even more reduced (0–19%). Although MSCs have lower concentration in CB than in marrow, they have a greater potential for proliferation (Bourin et al. 2008).

2.2.5. Multipotent Differentiation Potential:

MSCs exhibit multipotent differentiation potential and have been shown to give rise to different mesodermal cell lineages, including osteoblasts, chondroblasts, and adipocytes. MSC are also capable of differentiating into myocytes, cardiomyocytes, and even nonmesodermal origin cells, such as hepatocytes and neurons (Gimble et al. 2008).

It has to be pointed out that MSC preparations from different species (human, mouse, rat) and sources (BM, adipose tissue or CB) may vary in their surface epitope pattern, differentiation or proliferation capacity (Rojewski et al. 2008). For example, MSCs isolated from CB has broader potential of differentiation and have been found to be able to differentiate not only in the mesodermal tissues but also in the endodermal and ectodermal pathway (Caplan 2007). In general MSC that are found in fetal fluids such as amniotic liquid, placenta or Wharton’s jelly are characterized by having greater proliferation and differentiation potential than other MSCs (Bourin et al. 2008). Also, MSC populations of different donors vary in their growth properties and differentiation potential (Stolzing et al. 2008).
2.2.6. Transdifferentiation (phenotypic plasticity):

It is stated that MSC can, after fully committing to one lineage, are able to de-differentiate back to its original uncommitted state or trans-differentiate into another lineage after switching the inducing culturing medium (Caplan 2007). hMSCs cultured under osteogenic conditions for 10, 20, or 30 days maintained their ability to differentiate into adipocytes and chondrocytes. They were able to change their differentiation program after changing the induction medium and became lipid-producing adipocytes and chondrocytes that produced sulfated proteoglycan, collagen type II, and link protein (Song & Tuan, 2004).

![Diagram](image_url)

Figure 2.6: The Mesengenic process diagram depicting the plasticity of mesenchymal cells and the transdifferentiation (red arrows) of mature phenotypes into wholly different cell types (Caplan 2005).
Also, hMSCs that had differentiated into adipocytes after 20 days were able to transdifferentiate into osteoblasts or chondrocytes. Similarly, chondrocytes derived from MSCs induced in serum-free, 3-dimensional alginate culture in the presence of TGF-β3 could be dissociated and subsequently induced to differentiate into osteoblastic cells and adipocytes. Further support for this transdifferentiation was shown through the detection of the respective lineage-specific mRNA transcripts by RT-PCR (Song & Tuan, 2004).

Even fully differentiated MSC-derived cells could resume cell proliferation, modify their gene expression profile, and return to a more primitive stem cell-like stage (Kassem et al. 2008). When hMSCs were cultured in osteogenic medium for 30 days, adherent cells formed large osteoblastic nodules. When the osteogenesis-inducing medium was replaced with control medium, spindle shaped fibroblast-like cells started to migrate out of the calcified matrix nodule and were morphologically similar to the original hMSCs. The dedifferentiated cells derived from the osteoblasts not only exhibited similar morphology as MSCs, but also exhibited MSC-like multi-differentiation potentials (Song & Tuan, 2004).

2.2.7. MSC Uses:

MSCs are ideal candidates for use in regenerative medicine and tissue engineering, including gene therapy, cell replacement therapies, and cancer therapeutics (Choumerianou et al. 2008). This is due to their ability to differentiate to different phenotypes, and their secretion of bioactive macromolecules that attract endogenous repair mechanisms such as stimulating angiogenesis, and proliferation of progenitor cells,
inhibit scarring and limit apoptosis in the field of injury (Caplan 2007). Significant clinical improvement was seen through their use in the treatment of various diseases such as osteogenesis imperfecta, lung injury, kidney disease, diabetes, myocardial infarction and neurological disorders (cerebral ischemia) and other diseases of the central nervous system, including neurodegenerative and inflammatory disorders (Schäfer and Northoff 2008).

Examples of MSCs use; in one study, adipose tissue derived MSCs were investigated as alternative therapy for liver dysfunction. They were differentiated into hepatocytes, which were then transplantated into mice and incorporated into the parenchyma of the liver (Banas et al. 2007). Another application of MSCs that was recently reported; it was shown that autologous application with fibrin spray accelerated wound healing in patients with skin cancer and in patients with chronic, long-standing, non-healing lower extremity wounds (Choumerianou et al. 2008). Bone marrow MSCs cultured on calcium phosphate porous ceramics have been used in long-bone defect sites and results showed the production of morphologically and biomechanically superior bone (Bruder et al. 1998). In a similar study they were delivered on hydroxyapatite ceramic (HAC) carriers and demonstrated faster bone repair as compared to HAC alone (Kon et al. 2000). They were also used in hyaluronan and polymeric scaffolds for cartilage repair (Solchaga et al. 2005).

MSCs are used in the management of treatment-resistant graft-versus-host disease and other human immunological diseases, due to their impressive immunomodulatory
capacities. They exert regulatory activity on effector cells of both the innate and adaptive immune system, in addition to their role in hematopoiesis and lymphopoiesis (Bieback 2008). Their immunesuppressive role is expressed through their interactions with T and B cells of the immune system where T-cells proliferation and function is suppressed, B-cells proliferation, differentiation and chemotaxis is inhibited. Moreover, it was reported that they can suppress allospecific antibody production in vitro (Schafer & Northoff, 2008).

2.3. Bioactive Peptides:

2.3.1. Background:

The ability of cells to adhere to an extracellular material, proliferate and organize ECM molecules into a functional tissue is very crucial for new tissue formation (Miyamoto et al. 1998). One of the bases for constructing successful scaffolds in tissue engineering is to mimic the dynamic interactions between cells and the extracellular matrix in vivo. The incorporation of chemical cues such as adhesion ligands and growth factors that mimic the ones in the ECM into the engineered materials can give control over the cellular responses enabling the manipulation of the cellular pathway of actions (Maskarince & Tirrell 2005).

Current understanding of the biology and biochemistry of cellular function and differentiation can be utilized toward biochemical surface modifications of biomaterials. Major advances have been made in understanding the role of biomolecules in regulating differentiation and remodeling of cells and tissues. For example: the mechanisms by
which cells adhere to substrates have been under focus in a lot of research and became very much well understood which gave the ability to render different surfaces cells adhesive. These finding have led to acquiring the ability to do biochemically surface modification such as: immobilize proteins, enzymes, or peptides on biomaterials for the purpose of inducing specific cell and tissue responses and controlling the cellular interactions at and away from the tissue implant interface (Morra 2006).

The development of biomaterials for tissue engineering applications has recently focused on the design of biomimetic materials that are able to interact with surrounding tissues by biomolecular recognition. The design of biomimetic materials is build on the need to make the materials capable of eliciting specific cellular responses that are induced by specific interactions and hence directing new tissue formation (Shin et al. 2003). The biomimetic materials potentially mimic many roles of ECM in tissues. For example, the immobilization of signaling peptides renders the surface of biomaterials cell adhesive that inherently have been non-adhesive to cells (Shin et al. 2002). Moreover, the incorporation of peptide sequences into materials can also induce cellular responses that may not be present in a local native tissue (Susuki et al. 2000).

2.3.2. Cell-Biomaterial Interaction:

Cell recognition of implanted biomaterials in vivo occurs indirectly through the unspecific adsorption of proteins from body’s fluids onto the surfaces of these materials, and then cells indirectly interact with the biomaterial surface through these adsorbed proteins (Shin et al. 2003). Some of the proteins adsorbed, including fibronectin,
vitronectin, and fibrinogen that are naturally found in the ECM promote the adhesion of cells via cell-surface adhesion receptors for these proteins (Morra 2006). Other adsorbed proteins or growth factors, may affect the cells fate and function along the cells-material interface.

2.3.3. Ways of Materials Functionalization:

Directing new tissue formation mediated by specific interactions, can be manipulated by controlling the surface or bulk chemistry of the material altering instead of by non-specifically adsorbed ECM proteins. Biomolecular recognition of materials by cells can be achieved by two major design strategies. The first approach is through the incorporation of soluble bioactive molecules such as growth factors and plasmid DNA into the scaffold materials that are released from the material afterward to trigger or modulate the cellular responses and hence the type or structure of the tissue formed. The other approach involves the grafting of peptides into or on the biomaterials via chemical or physical means that are recognized by the cells receptors and can modulate the cellular responses (Sakiyama-Elbert & Hubbell, 2001).

2.3.4. Advantages of Using Peptides for Surface Modifications:

A simple peptide of a specific short amino acid sequence of few hundred Daltons can mediates cellular response similar to its considerably larger parental molecule of multiples of a hundred thousand Daltons (Morra 2006). It has been shown that synthetic peptides that contain the amino acids RGD, such as GRGDSP (Gly-Arg-Gly-Asp-Ser-
Pro), which are short primary sequences taken from the receptor-binding domains of adhesion proteins such as laminin and fibronectin, can essentially mimic cell attachment activity of the parental molecule (Pierschbacher & Ruoslahti, 1984). Cellular recognition of simple peptides suggests their potential usefulness of conveying particular cell adhesion properties to a material surface, thus enhancing cell-material interactions. Peptide linking to materials surfaces has since then recognized as a way for enhancing cell interactions with biomaterials (Massia and Hubbell, 1990).

Figure 2.7: Representation of the chemical structure of the amino acid and polypeptide, and aggregate of multiple proteins (Castner & Ratner 2002).

Other advantages of using small peptides rather than whole proteins are that they are relatively inexpensive to make and easy to purify, whereas whole adhesion proteins, such as fibronectin or laminin, are expensive and often not available in a clinically acceptable form (Sakiyama-Elbert & Hubbell, 2001). Peptides can be produced synthetically, allowing precise control of their chemical composition and avoiding issues related to concerns on proteins from animal sources. In addition, peptides are more
resistant to denaturing conditions such as variations in pH and heat, relatively to the big protein molecules (Morra 2006).

2.3.5. Factors to Consider in Peptides Surface Modification:

In order to use short peptides to render materials bioactive, several factors have to be considered for the fabrication of a successful bioactive material. One of these factors is the amount of peptides required for biological recognition to occur. In a study by Massia & Hubbell, they reported that for inducing cell adhesion and focal contact formation on poorly adhesive glass substrates, approximately $10^5$ copies per cell of the tri-peptide RGD had to be present on the surface. That with a peptide density equivalent to a 140 nm spacing between peptides for focal contact formation. They also found that this number was much more less than the amount of the whole protein fibronectin molecule to be used if they were allowed to be adsorbed on the substrate surface. That was attributed to the unfolding of some of the protein molecule upon adsorption in an orientation such that the receptor-binding domain was not accessible for binding (Messia & Hubbell, 1991).

The selection of specific adhesive types of peptides can control the type of cells that will adhere to the substrate. This is important useful when certain type of cells is required to adhere to the material from a pool of different cells. For example in vascular grafts, it is crucial that only endothelial cells adhere to the graft material and not the platelets (Sakiyama-Elbert & Hubbell, 2001). Also the spatial distribution of the peptides in the material whether it is in a two or three dimensional form will affect the cells distribution.
For immobilized synthetic peptides on biomaterial surfaces to maintain their biological activity, they should be designed to be flexible and experience minimal steric hindrance when grafted. The spacer linking the peptide to the surface should be inert, with certain molecular weights and non-specific repetitive (Shin et al. 2003). Hern et al. reported that when non-adhesive hydrogels were modified with RGD peptide with a polyethylene glycol peptide (PEG) spacer (MW 3400) at a low surface density (0.01 pmol/cm$^2$), cell adhesion was induced. While when the RGD peptide was used without the PEG spacer limited cell adhesion was induced even when higher surface density (1 pmol/cm$^2$) was used (Hern & Hubbell, 1998).

2.3.6. Applications of Peptides Modified Materials:

The incorporation of cell-type selective adhesion peptides in a biomaterial can enable the control of the cell type that adheres to the material surface. This through the targeting of the adhesion ligands that interact with cell adhesion receptors expressed selectively on the surface of the target cell population. For example, in the case of vascular graft design, Materials that support the adhesion and migration of endothelial cells but prevent the adhesion of blood platelets can be developed. By using the tetra-peptide adhesion ligand from fibronectin, REDV, which is specific for endothelial cells receptors and does not bind to other integrins, one can selectively target the adhesion of endothelial cells, but not platelets, to a biomaterial (Hubbell et al. 1991).

Peptides modified materials can be used for enhancing the adhesion and function of bone cells. Studies on the effect of surface linked peptides on osteoblast behavior showed...
that the adhesion peptide sequence RGDS (Arg-Gly-Asp-Ser) immobilized to surface-aminated glass enhanced the osteoblast adhesion on the initial hours of interaction. In the same study, growth factor osteopontin-1 was used in conjunction to the adhesion peptide and resulted in synergistic enhancement of the mineralization process (Dee et al. 1996). In another study, titanium rods coated with RGD were implanted into a Sprague Dawley rat long bone defect model of 1.64mm in diameter and 15mm in length in tibia. After 4 weeks of implantation, it was shown that newly formed bone coated the RGD modified titanium implant with greater thickness than the control group. This result shows the effect of adhesion peptide on enhancing the bone formation and integration of implants (Ferris et al. 1999).

Chemically conjugated peptides to silane precursors can be incorporated in silica sol-gel thin films for the use in devices where control on the cell’s interactions is required. In an experiment to induce neural differentiation of embryonic carcinoma cells, different combinations of adhesive and neural inductive peptides were added to a sol of silica precursor where subsequent gelation was induced. The results showed that the peptides were successfully expressed on the thin films and enhancement of the adhesion and neural differentiation was achieved (Jedlicka et al. 2007).
3.1. Introduction:

The use of simple molecules, such as small peptides that represent the active sites of larger proteins, are an excellent tool to fabricate platforms that mimic the extracellular matrix (ECM) (Maskarinec & Tirrell 2005, Shin et al. 2003). In the work presented here, three peptide sequences were chosen and synthesized in house. These peptides were chosen for their known activity in promoting the adhesion and osteogenic differentiation. One peptide contains the Arginine-Glycine-Aspartic Acid (RGD) domain that is found in several ECM proteins (Ruoslahti & Pierschbacher, 1987) and the other two peptides contain two different domains from the BMP-2 (BMP = Bone Morphogenetic Protein) and BMP-9 (Celeste et al. 1990, He et al. 2008, Bergeron et al. 2007, Zouani et al. 2010). These three peptide sequences were chosen to modify the surface of bioactive glass materials.

Bioactive glasses have attracted great interest in the field of bone regeneration as they almost fulfill the required criteria for bone scaffolds (Jones 2008). In the study reported here, we chose to prepare bioactive glass in the composition of 70% SiO$_2$–30% CaO (in molar percentage) as this composition possesses the highest bioactivity among bioactive glass compositions through the fast formation of the hydroxyapatite layer (Saravanapavan et al. 2001). The sol-gel technique was utilized for the fabrication of the materials of the study. It was chosen for the feasibility it offers for incorporating bioactive molecules during the sol state and the control over the shape of the end
material. Two shapes of materials were fabricated, bioactive glass-gel thin films and 3D bioactive glass scaffolds. For the 3D Scaffolds, a polymerization-induced (spinodal-type) phase separation stage was incorporated in the steps of the synthesis. This method allows the fabrication of nano/macroporous bioactive glass scaffold (Marques 2009).

3.2. Materials Synthesis:

3.2.1. Peptides Preparation:

Peptides were synthesized using standard FMOC solid state synthesis on an Intavis Multi-Pep synthesizer as described in Jedlicka (2007). TentaGel™ S PHB-Ala-Fmoc (Fmoc-L-alanine 4-[poly(ethylenoxy)]benzyl ester polymer-bound) (Fluka) was used as the solid phase. Protected amino acids (Nova Biochem) were added to the growing peptide chain with the activating reagent 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluoro-phosphate (HBTU) (Anaspec, Inc.).

Upon the addition of the N-terminal amino acid, the FMOC group was removed with 20% piperidine (Anaspec, Inc). Then 1, 1-Carbonyldiimidazole (CDI) (Sigma) (0.3 M in HBTU) was added to the peptide chain to activate the N-terminus amino group. Following activation and dimethylformamide (DMF) washing, 0.3 M aminopropyltrimethoxysilane (APTMS) (Acros Inorganic) in HBTU was attached to the activated N-terminal. That was followed by another DMF and Ethanol washing, and then the peptides were left to dry in a desiccators overnight. Using a cleavage cocktail composed of 88% Trifluoroacetic acid (TFA), 5% Phenol, 5% H₂O and 2% Triisopropylsilane (TIPS), all peptides were cleaved from the resin and the protection
groups were removed from the side chains. Peptides were then purified using ether precipitation, followed by at least five ether washes prior to peptide drying, to remove a majority of the protective groups from the peptide-silanes prior added to sol gel synthesis. All solvents and remaining peptide synthesis chemicals were obtained from Sigma and EMD.

Table 3.1: Representation of the amino acids used.

<table>
<thead>
<tr>
<th>Amino Acid Abbreviation</th>
<th>Name</th>
<th>N-Terminal Protection Group</th>
<th>C-Terminal Protection Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Fmoc-Ala-OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Fmoc-Cys(Trt)-OH</td>
<td></td>
<td>Tripheylmethyl (Trt)</td>
</tr>
<tr>
<td>D</td>
<td>Fmoc-Asp(OtBu)-OH</td>
<td></td>
<td>tert butanol (OtBu)</td>
</tr>
<tr>
<td>E</td>
<td>Fmoc-Glu(OtBu)-OH</td>
<td></td>
<td>tert butanol (OtBu)</td>
</tr>
<tr>
<td>G</td>
<td>Fmoc-Gly-OH</td>
<td>9-Fluorenylethoxycarbonyl (Fmoc)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Fmoc-Ile-OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>Fmoc-Lys(Boc)-OH</td>
<td></td>
<td>t-Butyloxycarbonyl (Boc)</td>
</tr>
<tr>
<td>L</td>
<td>Fmoc-Leu-OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>Fmoc-Pro-OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>Fmoc-L-Ser(tBu)-OH</td>
<td></td>
<td>t-Butyl (tBu)</td>
</tr>
<tr>
<td>T</td>
<td>Fmoc-Thr(tBu)-OH</td>
<td></td>
<td>t-Butyl (tBu)</td>
</tr>
<tr>
<td>V</td>
<td>Fmoc-Val-OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>Fmoc-Tyr(tBu)-OH</td>
<td></td>
<td>t-Butyl (tBu)</td>
</tr>
</tbody>
</table>

Table 3.2: Peptides sequences used in the study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Origin</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD</td>
<td>AYAVTGRGDSPAS</td>
<td>Fibronectin</td>
<td>Type III repeat</td>
<td>Ruoslahti et al (1987)</td>
</tr>
<tr>
<td>CKI</td>
<td>ACKIPKASSVPTELSAISTLYL</td>
<td>BMP-2</td>
<td>Residues 73-92</td>
<td>Balasundaram et al (2007)</td>
</tr>
</tbody>
</table>
3.2.2. 2D Thin Films Preparation:

Bioactive glass in the composition of 70% SiO$_2$–30% CaO was prepared by sol-gel technique. Tetramethyl orthosilicate (TMOS, 2.25 mL) (Acros Inorganic) was dissolved in 5 ml 0.05N acetic acid, and stirred for 15 min. The peptide silanes were dissolved in a 50 µL drop of dimethyl sulfoxide (DMSO) (Sigma) suspended in buffer and added to a final concentration of 0.01% mole peptide per mole of SiO$_2$ and allowed to completely dissolve before adding 1.545 g calcium nitrate. While the solution is still in its sol phase, clean glass cover slips were dip-coated and the sol was allowed to gel on the glass slips at room temperature. Four sets of materials were prepared (Table 3.2), bioactive glass without peptides, with RGD, with RGD and CGG, and with RGD, CGG and CKI. All coated glass cover slips were UV sterilized and stored in phosphate buffer saline (PBS) in 4 °C until used.

Figure 3.1: Illustrated diagram representing the steps of sol-gel bioactive glass fabrication
Table 3.3: The different experimental samples of the 2D thin films.

<table>
<thead>
<tr>
<th>Abbreviated Name</th>
<th>Experimental Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>Sol Gel Only</td>
</tr>
<tr>
<td>RGD</td>
<td>SG + RGD</td>
</tr>
<tr>
<td>CGG</td>
<td>SG + RGD + CGG</td>
</tr>
<tr>
<td>CKI</td>
<td>SG + RGD + CGG + CKI</td>
</tr>
</tbody>
</table>

3.2.3. 3D Scaffolds Preparation:

3.2.3.1. Scaffolds Synthesis:

Bimodal porous bioactive glass in the composition of 70% SiO₂–30% CaO was prepared by sol-gel technique with polyethylene oxide used to induce spinodial phase separation. Briefly, 2.1 g water soluble polyethylene oxide (PEO), with an average molecular weight of Mw =100,000 was dissolved in 30 ml of 0.05 N acetic acid (CH₃COOH). The mixture was stirred for 20 minutes until the entire polymer was dissolved. Then 1.35g Urea was added, followed by adding of 13.5 ml of tetramethoxysilane (TMOS) (Acros, Inorganic) under vigorous agitation for 20 min. 9.27g of Calcium nitrate tetrahydrate (Ca (NO₃)₂·4H₂O) was then added and stirred for 10 minutes. To accelerate gelation, aqueous hydrofluoric acid (HF, 6%) was added and quickly the sol was poured in molds and left to gel at 40°C for 24 hours. The resulting gel was dried in an atmosphere with controlled relative humidity (RH = 90%) at 60°C for 1 day and at 180°C for 2 days; then, they were heat treated sequentially at 600°C (1h) and 700°C (2h), with heating and cooling (to room temperature) rates of 100°C/h.
3.2.3.2. Peptides Addition to 3D Scaffolds:

Peptides were prepared as described earlier in chapter 3 and were added with concentration of 0.01% mole peptide per mole of SiO$_2$ as calculated present in each of the specimens. Briefly, all samples were autoclaved sterilized and wetted with autoclaved DI-H$_2$O overnight before the addition of the peptides. DI-H$_2$O was used specifically (instead of PBS or stimulated body fluid) to limit the formation of the HA layer formation until the added peptides adsorb to the surface of the materials. The desired amount of each peptide was dissolved in 50 µl of dimethyl sulfoxide (DMSO) (Sigma) then further diluted in autoclaved DI-H$_2$O and mixed so that the required concentration and combination of peptides is obtained. The peptide solutions were then added to the samples and left for 24 hour to allow the peptides to bond to free –OH groups on the surface of the bioactive glass before adding culture media. All incubation periods and
procedure steps were done in an aseptic environment. Four sets of materials were prepared (Table 3.3).

Table 3.4: The different experimental samples of the 3D scaffolds.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Experimental Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIO</td>
<td>Bioactive Glass only</td>
<td></td>
</tr>
<tr>
<td>RGD</td>
<td>Bioactive Glass + RGD</td>
<td></td>
</tr>
<tr>
<td>CGG</td>
<td>Bioactive Glass + RGD + CGG</td>
<td></td>
</tr>
<tr>
<td>CKI</td>
<td>Bioactive Glass + RGD + CGG + CKI</td>
<td></td>
</tr>
</tbody>
</table>

3.3. Characterization:

3.3.1. MALDI-mass spectrometry:

MALDI-mass spectrometry analysis was used to confirm silane coupling through the expected m/z ratio. Briefly, matrix solution was prepared by dissolving α–cyano-4-hydroxycinnamic acid (HCCA) in a mixture of acetonitrile (ACN) and 0.1% trifluoro acetic acid (TFA) (volume ratio 1:2 respectively) up to saturation at room temperature. This mixture was then centrifuged and the transparent homogenous phase was saved as the matrix solution. Amount of RGD peptide and RGD peptide silane powders were dissolved in 125 µl of 0.1% trifluoro acetic acid (TFA) in separate eppendorf tubes and then equal volumes of the peptide solution and the matrix solution were mixed. 1 µl from each mix was applied to a standard steel target and allowed to dry at room temperature. The steel target was then introduced into Bruker Daltonics MALDI-TOF mass spectrometer and the peptides were tested.
3.3.2. X-ray Photoelectron Spectroscopy:

XPS surface analysis was performed to confirm the presence of the peptides on the surface and the successful grafting. Samples were dried in an oven overnight at 40 °C, then placed in an introduction chamber, pumped down to UHV, and then transferred, under UHV, onto the analysis chamber and loaded onto the sample stage. The XPS core level spectra were obtained using Scienta ESCA-300 spectrometer with monochromatic Al Ka X-rays (1486.6 eV).

3.4. Results:

3.4.1. MALDI-mass spectrometry:

Peptides and peptides conjugated to the silane molecule (APTMS) of the RGD sequence were analyzed by MALDI-MS for confirmation of the successful conjugation of the silane molecule. The molecular ions generated for each sequence product are within experimental limits of the expected nominal molecular mass, demonstrating a successful conjugation of the APTMS with the CDI linker.

![Representative diagram of the RGD peptide chemical structure.](image)

Figure 3.3: Representative diagram of the RGD peptide chemical structure.
Table 3.5: Peptides fragmentation peaks detected in MALDI spectra.

<table>
<thead>
<tr>
<th>Peptide sample</th>
<th>Nominal Molecular weight (g mol⁻¹)</th>
<th>Reported Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGD without silane</td>
<td>1322.38</td>
<td>1677.94 (13%), 1573.93 (17.4), 1321.78 (43.5), 1325.88 (29.6), 1161.43 (47.8), 1055.21 (39.1), 908.92 (100), 793.91 (56.5), 473.16 (30.4)</td>
</tr>
<tr>
<td>RGD with silane</td>
<td>1526.70</td>
<td>1676.27 (9.5%), 1572.38 (11.4), 1320.7 (21.9), 1159.84 (53.3), 1045.1 (42.9), 988.1 (38.1), 908.17 (100), 849.2 (57.1), 793.3 (71.4), 660.26 (58.1), 602.31 (45.7), 545.34 (55.2), 486.38 (49.5)</td>
</tr>
<tr>
<td>CGG with silane</td>
<td>2496.99</td>
<td>2446.35 (2.8%), 1848.41 (6), 1752.16 (16), 1750.2 (25), 1733.85 (4), 1680.87 (6), 1679.14 (11.2), 1565.78 (3.5), 1188.65 (3), 878.45 (19.6), 879.43 (10.5), 880.32 (5), 694.25 (11.2), 695.23 (5.6), 694.25 (11.2), 552.31 (24.5), 550.36 (100)</td>
</tr>
<tr>
<td>CKI with silane</td>
<td>2628.27</td>
<td>2811.48 (29%), 2809.75 (40.2), 2808.76 (34.1), 2807.75 (21.2), 2753.02 (30.3), 2752.88 (32.6), 2750.96 (23.5), 2692.9 (23.5), 2690.27 (39.4), 2688.82 (47.7), 2689.82 (59.9), 2632.63 (29.6), 2552.49 (23.5), 2491.29 (73.5), 2488.47 (40.2), 2490.48 (100), 2434.09 (36.4), 2433.25 (30.3)</td>
</tr>
</tbody>
</table>
3.4.2. 3D Scaffold Preparation:

Bioactive glass porous disks were obtained with dimensions of 1 cm diameter. All disks were surface grinded to obtain uniform thickness of about 3 mm (Figure 3.4).

![Bioactive glass porous disk](image)

Figure 3.4: Bioactive glass porous disk.

3.4.3. X-ray Photoelectron Spectroscopy:

XPS surface analysis of the samples showed the presence of the Si, Ca, O peaks in the spectra (Figure 3.5) as the compositional elements of the material, along with the presence of peaks for Carbon and nitrogen in the spectra of the grafted samples (Figure 3.6). This confirms the presence of the peptides on the surface of the scaffolds and the successful attachment of the peptides to the surfaces. In addition, the atomic percentages from the spectra were calculated and the Nitrogen : Carbon ratio was compared to their ratio from the expected molecular structure (Table 3.6). Sodium and fluoride appeared in the spectra are impurities from the agents used in the synthesis (e.g. trifluoroacetic acid used in the cleavage step of the peptides).
Table 3.6: Elemental atomic percentages and N : C ratio from XPS survey spectra.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Si 2p (%)</th>
<th>Ca 2p (%)</th>
<th>Na 1s (%)</th>
<th>O 1s (%)</th>
<th>C 1s (%)</th>
<th>N 1s (%)</th>
<th>F 1s (%)</th>
<th>N/C ratio from XPS survey spectra</th>
<th>N/C ratio from proposed structural</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIO</td>
<td>26.70</td>
<td>1.59</td>
<td>0.63</td>
<td>66.79</td>
<td>4.26</td>
<td>0.02</td>
<td>0.00</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RGD</td>
<td>28.63</td>
<td>1.61</td>
<td>0.22</td>
<td>65.33</td>
<td>3.29</td>
<td>0.46</td>
<td>0.46</td>
<td>0.14</td>
<td>0.34</td>
</tr>
<tr>
<td>CGG</td>
<td>25.34</td>
<td>2.56</td>
<td>0.07</td>
<td>67.63</td>
<td>3.67</td>
<td>0.19</td>
<td>0.55</td>
<td>0.05</td>
<td>0.30</td>
</tr>
<tr>
<td>CKI</td>
<td>21.74</td>
<td>2.42</td>
<td>0.03</td>
<td>54.16</td>
<td>17.92</td>
<td>3.28</td>
<td>0.45</td>
<td>0.18</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Figure 3.5: XPS spectra of the bioactive glass without grafted peptides.
Figure 3.6: XPS spectra of the CKI sample (bioactive glass with the three peptides).
3.5. Discussion:

Peptide usage in biomaterials to provide guidance control over the cells interaction has revolutionized the field of tissue regeneration. These synthetic molecules can provide specific bioactive signals to control the biological environment around the implanted material during the healing process and achieve excellent outcomes (Mikos et al. 2006). Peptides are easily synthesized with the desired sequence which offer another advantage of these molecules in controlling their chemical and physical properties through the manipulation of the order of the amino acids sequences for the achievement of the desired conformation, linking ability and the signal they should provide to the cells (Morra 2006).

The study here focuses on the differentiation of stem cells toward the osteogenic lineage with the use of specific peptides as the inductive molecules while they are covalently linked to a substrate. The 3 peptide-silanes synthesized here in this study where chosen based on that concept. The RGD sequence comes mainly from the fibronectin protein and it was used extensively in research to promote the adhesion of cells on materials (Ruoslahti 1996). The CGG and CKI sequences are found in BMP-9 and BMP-2 respectively, which are members of the transforming growth factors-b family and are well known for their ability to induce bone formation in-vitro and in-vivo. In general BMPs are key regulators of cellular growth and differentiation, and regulate tissue formation in both developing and mature organisms (Hogan 1996).
After the synthesis of the peptides and before their usage in our experiment, RGD Peptide-silanes and RGD free-peptide samples were analyzed with MALDI-MS for the confirmation of the successful linkage of the silane group to the peptide chain through the comparison of the mass-to-charge (m/z) ratios of their fragmented ions. Data showed that peptides tested were of the expected molecular mass which confirms the successful conjugation of the silane group (APTMS molecule) and the CDI linking molecule to the peptide chain. Few of the fragmented ion showed higher molecular mass than the nominal mass of the peptides molecules that may refer to incomplete removal of the protecting groups but the intensity of the peaks were very small and insignificant. The data is also in agreement with the reference study that the technique was adapted from it (Jedlicka et al. 2007) which further confirms it.

Bioactive glass as an attractive scaffold for bone regeneration due to its high bioactivity and its induction effect on the bone formation process was chosen here to further try to increase its inductive effect for better and faster bone regeneration. Functionalized thin films of bioactive glasses can be applied as coating to metal implants for better integration and bone formation along the interface (Gabbi et al. 1995). 3D forms of the bioactive glass with specific architects are being developed for bone tissue engineering and many studies are being done to functionalize the surface of these scaffolds for controlling the spatial growth of the cells and their phenotype (Chen et al. 2006, Zhang et al. 2009).
Bioactive glass thin films and 3D porous scaffolds were prepared in the study here. The 3 Peptides that were synthesized as mentioned earlier and were incorporated during the synthesis of the thin films in different combinations to be presented on its surface. For the 3D forms, the peptides were allowed to adsorb to the surface after the complete synthesis of the bioactive glass samples as they would not have survived the high temperatures used in the synthesis process of the bioactive glass 3D scaffolds. The adsorption and bonding of the peptides to the surface of the 3D forms was confirmed by XPS. The XPS data showed the presence of nitrogen and carbon on the surface of the samples which can be attributed to the presence of the peptides and their successful coupling to the surface (Jedlicka et al. 2007).

These materials will be tested with hMSCs to investigate the ability of the peptides to enhance the inductive effect of the bioactive glass on the osteogenic differentiation of the hMSCs. There are debates in the literature about the effect of the bioactive glass on the hMSCs. It is unclear if the induction effect that the bioactive glasses induce on the osteoblastic progenitor cells, also apply on the hMSCs. Some studies reported that the bioactive glass has no direct inductive effect on the hMSCs differentiation (Leach et al. 2006, Reilly et al. 2007), while others reported the differentiation of the hMSCs to bone cells after culturing on the bioactive glass (Karpov et al. 2008, Sollazzo et al. 2010). The development of a defined material that support the adherence and osteogenic differentiation of the hMSCs will give great impact in the bone tissue engineering field.
Chapter 4: Human Mesenchymal Stem cells culture and characterization

4.1. Introduction:

Human Mesenchymal Stem cells (hMSCs) were utilized in this study as they have the ability to undergo osteogenesis (Pittenger et al. 1999, Minguell et al. 2001). They are routine to culture and expand, and the osteogenic pathway is relatively well understood. In addition, these cells are potentially clinically relevant in the field of tissue regeneration for their efficacy in the modulation of immune disorders such as graft versus host disease and their role in establishing the marrow microenvironment both in vitro and in vivo. hMSCs were characterized here for their stemness state and their ability to differentiate to different lineages.

4.2. Experimental:

4.2.1. hMSCs culture:

hMSCs (Lonza, USA) were cultured using mesenchymal stem cell growth media (MSCGM) (Lonza, USA) on 10 cm tissue culture polystyrene plates (VWR). Cells were maintained in 5% CO₂ at 37°C in a humidified atmosphere. Media was changed twice a week and cells were passaged when cells reached 80% confluency.

4.2.2. Osteogenic Differentiation:

hMSCs passage 4 were seeded in 4 glass bottom 3.5 cm diameter sterile dishes in MSCGM until they reached 70-80% confluency, then differentiation media composed of
DMEM/low glucose, 10% FBS (hMSC special), 0.1 uM dexamethasone, 10 mM β-glycerol phosphate and 0.05 mM Ascorbic acid was used. Media was changed twice a week.

4.2.3. Adipogenic Differentiation:

hMSCs passage 4 were seeded on sterile glass coverslips in a 12 well plate and cultured with the mesenchymal stem cell growth media (MSCGM) to confluency. After cells reached 100% confluency, the media was changed to Lonza induction media for 3 days and then changed again to Lonza maintenance media for 4 days. This induction/maintenance cycle was repeated for 4 weeks, and then cells were fixed.

4.2.4. Myogenic Differentiation:

hMSCs passage 10 were seeded on glass coverslips in a 12 well plate at a density of 10,000 cells per well and 5 µl of 1mM Azacytidine were added to each well with 1ml of Lonza basal media in each well, to have a final concentration of 5 µM. After 24 hours the media was changed with the basal media without the Azacytidine. This treatment was done once a week up to 4 weeks. Cells were fixed on the 1st, 2nd, 3rd, and 4th weeks.

4.2.5. Chondrogenic Differentiation:

hMSCs passage 6 were suspended in 0.5 ml complete chondrogenic media (Lonza) in sterile 15 ml tubes with cell density of 5 x 10^5 cells/ml. Then cells were centrifuged at 150x g for 5 minutes at room temperature to form a cell pellets. Tubes were incubated at
37°C, in a humidified atmosphere of 5% CO₂, cell pellets were not disturbed for the first 24 hours. Next day tubes were shook to make the pellets free floating. Media changed every 3 days.

Two pellets were fixed at 1, 2, 3 and 4 weeks, cryopreserved by embedding into OCT embedding media and frozen by placing on top of liquid nitrogen for 30 seconds followed by freezing at -80°C. The frozen blocks were later sectioned and immunocytocheemistry stained for the expression of collagen II protein.

4.2.6. Neurogenic Differentiation:

hMSCs passage 4 were seeded in 12 well non-adherent plate at a density of 2x10⁵ cells per cm² and cultured in P4-8F media supplemented by 20 ng/ml of both Epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) at 37°C, in a humidified atmosphere of 5% CO₂ for 8 weeks. Media was changed once a week and growth factors were added twice a week. Neurospheres formed after 2 weeks.

At the 8th week, neurospheres were seeded on UV sterile poly-d-lysine coated glass coverslips and cultured for 2 weeks in neurobasal media supplemented by 0.5 umol/L all-trans-retinoic acid, 1% fetal bovine serum (FBS), 5% horse serum, 1 % N2 supplement and 1% penicillin/streptomycin. To induce glial differentiation, 10 ng/ml rh-PDGF-BB was added to the media. Media changed twice a week.
4.3. Characterization:

4.3.1. Phase Contrast Imaging:

Phase contrast images were taken every week to follow the changes in cells morphology during differentiation. Imaging was conducted on a Zeiss Observer Z1.

4.3.2. Colorimetric Staining:

4.3.2.1. Alizarin Red Staining:

Alizarin Red dye is used to identify calcium in tissue sections. Calcium forms an Alizarin Red-calcium complex in a chelation process, and the end product is birefringent. Alizarin red stain solution was prepared and its pH was adjusted to 4.25 with Ammonium Hydroxide. Osteogenic differentiated Cells were fixed with 10% formalin, and treated with the stain solution for 30 min at room temperature. Then cells were washed with 1x PBS and imaged.

4.3.2.2. Oil Red O Staining:

Oil Red O dye is used to detect lipid formation in cells and tissues as it is more soluble in lipids. A stock solution was prepared by dissolving 30 mg of Oil Red powder in 10 ml of 100% isopropanol. For the working solution; 3 parts of stock solution were mixed with 2 parts of DI-water and filtered. The adipogenic differentiated cells were fixed with 10% buffered formalin, and treated with 60% isopropanol for 5 min. Isopropanol was then removed and the Oil Red O working solution was added for 5 min at room temperature. Nuclei were stained with Hematoxylin stain.
4.3.3. Immunocytochemistry:

Cells were fixed with 10% buffered formalin for 15 min. This was followed by 3 x 2 minutes rinsing with 1xPBS. The samples were then treated with 100% methanol for 7 min, followed by permeabilization with 0.1% Triton for 15 minutes, and blocking for non-specific binding with 10% FBS or 1% BSA. Samples were reacted with specific primary antibodies according to each differentiation group (Table 4.1). After incubation overnight at 4 °C cells were treated with appropriate secondary antibodies (Table 4.1) as necessary for 1 h at room temperature. The samples were then rinsed (3 x 2 minutes) with 1x PBS and the nuclei were counterstained with Hoechst dye (0.002 mg/ml in 1x PBS) for 5 minutes. The samples were then rinsed (2 x 2 minutes) with ddH2O prior to imaging. Fluorescent microscopy was conducted on a Zeiss Observer Z1.

Table 4.1: Representing the cell types and the markers used with them.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Marker</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSCs</td>
<td>CD44 (FITC Conjugate)</td>
<td>Anti mouse IgM (Alexa Fluor 555)</td>
</tr>
<tr>
<td></td>
<td>Stro-1</td>
<td></td>
</tr>
<tr>
<td>Osteogenic cells</td>
<td>Osteopontin</td>
<td>Anti mouse IgG (Alexa Fluor 546)</td>
</tr>
<tr>
<td></td>
<td>Osteonectin</td>
<td>Anti mouse IgG (Alexa Fluor 546)</td>
</tr>
<tr>
<td></td>
<td>Osteocalcin (Phycoerythrin Conjugate)</td>
<td></td>
</tr>
<tr>
<td>Adipogenic cells</td>
<td>ALK-7</td>
<td>Anti-Rabbit IgG (FITC)</td>
</tr>
<tr>
<td></td>
<td>aP-2</td>
<td>Anti mouse IgG (Alexa Fluor 546)</td>
</tr>
<tr>
<td>Chondrogenic cells</td>
<td>Collagen II</td>
<td>Anti mouse IgG (Alexa Fluor 546)</td>
</tr>
<tr>
<td>Myogenic cells</td>
<td>Myocin Heavy Chain</td>
<td>Anti mouse IgG (Alexa Fluor 647)</td>
</tr>
<tr>
<td></td>
<td>Troponin-T</td>
<td>Anti mouse IgG (Alexa Fluor 555)</td>
</tr>
<tr>
<td></td>
<td>Tropomyosin</td>
<td>Anti sheep IgG (Cy3)</td>
</tr>
<tr>
<td>Neurogenic cells</td>
<td>CNPase,</td>
<td>Anti mouse IgG (Alexa Fluor 488)</td>
</tr>
<tr>
<td></td>
<td>GFAP (Cy3 Conjugate)</td>
<td></td>
</tr>
</tbody>
</table>
4.4. Results:

4.4.1. hMSCS Stemness Markers:

hMSCs used in the experiments did express CD44 and STRO-1 stem cell markers prior to any induction into other lineage. (Figure 4.1)

![Figure 4.1: ICC images showing the expression of Stro-1 (left) and CD44 (right).](image)

4.4.2. Osteogenic Differentiation:

Differentiation of the hMSCs to the osteogenic lineage was done and cells showed change in morphology and distribution as seen in Figure 4.2, where cells gathered and grouped together forming distinctive patterns by day 18. Mineralization was shown by the Alizarin Red colorimetric dye as it stain orange-red in the presence of calcium. (Figure 4.3)
Differentiation was further confirmed by the expression of 3 osteogenic markers, osteopontin, osteonectin and osteocalcin, representing early, middle and late respectively stages of differentiation (Figure 4.4). All 3 proteins were expressed by the cells beside the formation of mineralization nodules as seen in the osteocalcin images.
Figure 4.4: Immunofluorescent images construct showing the expression of the 3 osteogenic proteins at day 21 for Osteopontin and day 37 for Osteonectin and Osteocalcin.
4.4.3. Adipogenic Differentiation:

hMSCs induced to differentiate to lipid cells showed the formation of the lipid vacuoles after the first cycle of induction at week 1 (Figure 4.5 (a)). In the successive weeks the vacuoles became bigger in size (Figure 4.5 (b)). The presence of the lipid droplets were further conformed by staining with Oil Red O that stained the lipid vacuoles with red color (Figure 4.6).

Figure 4.5: Phase contrast images showing the formation of the lipid vacuoles. (a) Week 1, (b) week 2.

Figure 4.6: Oil Red O staining of the lipid vacuoles formed in the differentiated hMSCs.
Activin receptor-like kinase 7 (ALK7), a receptor for TGF-β family members and is reported to have a role in regulation of metabolism and adipose tissue function (Carlsson et al. 2009), and adipocyte protein 2 (aP2) a carrier protein for fatty acids, are proteins that are expressed in adipocytes. To further confirm the differentiation of the hMSCs to the adipogenic phenotype, these two proteins were investigated for their expression. At week 1, neither of these 2 proteins was expressed. By week 2, aP2 started to be expressed while ALK7 was detected by week 3. Both proteins showed enhanced expression by week 4 (Figure 4.7).

![Figure 4.7: ICC images showing the expression of ALK7 and aP2 proteins at week 1 and 4.](image-url)
4.4.4. Chondrogenic Differentiation:

Thin sections were cut by the use of a cryotome from the frozen blocks of the embedded chondrogenic differentiated pellets and fixed on clean glass slides. Slides stained for collagen II showed expression of this protein (Figure 4.8) confirming the differentiation of the cells and the secretion of this protein in the matrix of the pellet tissue (Figure 4.9).

Figure 4.8: Phase contrast image of thin section of the chondrogenic differentiated cell pellet.

Figure 4.9: ICC image showing the expression of collagen II in thin section from the chondrogenic differentiated cell pellet at week 4.
4.4.5. Myogenic Differentiation:

hMSCs differentiated into the myogenic phenotype showed cells arrangement parallel to each other with the alignment of the actin fibers parallel to the long axis of the cells, in addition to cell elongation in the direction of alignment (Figure 4.10 (b)). A periodic grouping of the cells was also noticed that was perpendicular to the direction of the alignment of the cells (Figure 4.10 (c, d)).

Figure 4.10: Phase contrast images showing (a) control undifferentiated cells, (b) alignment of the cells and actin fibers, (c) and (d) the periodic grouping perpendicular to the cell alignment (arrows).
Cells were examined using immunocytochemistry for 3 different myogenic markers, Myosin Heavy Chain (MHC), Tropomyosin (TM) and Troponin T (TT). MHC and TT were detected from week 1 till week 4, while TM was detected from week 2. Control cells not induced for myogenic differentiation were tested for the expression of the same 3 proteins and showed negative expression for the TM and TT, while positive expression for MHC.

Figure 4.11: ICC images showing MHC expression week 1 (left) and week 4 (right)

Figure 4.12: ICC images showing Troponin T expression week 1 (left) and week 2 (right)
4.4.6. Neurogenic Differentiation:

After cells were seeded in low attachment wells, they started to aggregate and form small nodules (Figure 4.14 (left)). By the 2-3 week small spheres (neurospheres) were formed and were free floating in the culture media (Figure 4.14 (right)). After seeding the neurospheres on the poly-D-lysine coated coverslips, cells started migrating out of the spheres (Figure 4.15).

Figure 4.14: Phase contrast images showing (a) cells forming small nodules, (b) neurospheres floating in the culture media.
Adhered cells to the poly-D-lysine coated coverslips were examined for the expression of CNPase enzyme (2', 3'-cyclic-nucleotide 3'-phosphodiesterase) which is an enzyme specifically seen in oligodendrocytes and Schwann cells, and its expression is one of the earliest indication for the oligodendrocyte differentiation (Kasama-Yoshida et al. 1997). Also cells were examined for the expression of GFAP (Glial fibrillary acidic protein) which is an intermediate filament (IF) protein that is expressed by the central nervous system (CNS) cells and it is reported that it has a role in cell structure stability, cell communication and maintenance of CNS myelin integrity (Eng et al. 2000, Goss et al. 1991). Control undifferentiated cells were also examined. Both control and differentiated cells did express the two markers as seen in Figure 4.17 for the co-staining of both markers. Although the CNPase enzyme is expressed by both control and differentiated cells, there was different distribution pattern of the enzyme in both types of cells. Differentiated cells show diffuse distribution of the enzyme in the cell body with the formation of discrete vacuoles or droplets containing concentrated amounts of this
enzyme around the cell bodies. While in the undifferentiated cells the enzyme seems to be confined to the endoplasmic reticulum inside the cells (Figure 4.16).
Figure 4.16: ICC images showing comparison between control and differentiated samples for the expression of CNPase protein.
Figure 4.17: ICC images showing comparison between control and differentiated samples for the co-expression of CNPase + GFAP protein.
4.5. Discussion:

MSCs are self renewal multipotent progenitor cells that can differentiate into multilineages. Culturing conditions, passage number, site of isolation (where the cells came from) and donor age can affect the capacity of the cells to differentiate. Cells in culture lose some of their capacity to differentiate (their stemness) as they get older (high passage number) and same if they came from an old donor versus a young one (Bourin et al. 2008). For the MSCs to retain this differentiation capacity to multiple types of tissues, they should be expanded and maintained in their primary undifferentiated state.

hMSCs were tested for their expression of two surface markers (CD44, STRO-1) that are only expressed in undifferentiated cells to confirm that they are uncommitted to a particular lineage (Rojewski et al. 2008). Results revealed that the cells were expressing these two markers and therefore should be retaining their capacity for multilineage differentiation. In addition, differentiation experiments were carried out to confirm their ability to differentiate to bone, fat, muscle, cartilage and neurons, and to study the morphological changes that occur to the cells as they differentiate to each of the aforementioned lineage.

Osteogenic differentiation was carried out on the hMSCs by the use of well known protocol, using dexamethasone, beta-glycerolphosphate, and ascorbic acid as induction agents. The cells showed gradual changes in morphology and organization resembling the trabecular bone pattern in some sort but in 2D form (Pittenger et al. 1999). Confirmation of the osteogenic differentiation was carried out through detecting the morphological
changes to the cells shape by phase contrast microscopy along the course of the differentiation experiment. Also, testing for the expression of osteogenic markers (OP, ON, OC) showed their positive expression and increase of the expression as the cells went along the differentiation path. OP was the first to be detected from the first week of differentiation, followed by the ON and OC protein at the 3\textsuperscript{rd} week. The OC expression further increased at subsequent weeks as the mineralization process started and mineralized nodules started to appear within the cells sheet (Mizuno & Kuboki, 2001). Formation of mineralized tissue was confirmed by the alizarin red staining which showed positive staining showing the orange color characteristic of the reaction of the dye with the calcium phosphate mineral (Puchtler et al. 1969).

The adipogenic induction was accomplished through using of specific induction media (Lonza) and the differentiation of the hMSCs was manifested with the formation of the fat droplet in the culture plate as detected in the phase contrast images (Sekiya et al. 2004). These droplets were also stained positive for the oil red O stain that interacts with triglyceride and lipids in tissues to give a reddish color, thus confirming the accumulation of these molecules in the formed droplet and confirming the identity of the droplet. Cells were also stained for two markers (ALK7 and aP2) specifically expressed in fat cells and showed positive expression at the 2\textsuperscript{nd} week for aP2 and at the 3\textsuperscript{rd} week for ALK7 while there was increase of expression for both by the 4\textsuperscript{th} week. These results are in agreement with other studies (Kogame 2006) as indicated that ALK7 is a late marker that appear in late stages of adipogenic differentiation, justifying its appearance on the 3\textsuperscript{rd} week of the induction process.
hMSCs were also induced to differentiate to muscle cells by the use of weekly doses of 5-Azacytidine (cytosine analog) as differentiation agent. The mechanism of 5-azacitidine effect on differentiation is still unclear. It is characterized by demethylating activity and, according to published data, is indicated for directed differentiation of MSC into cardiomyocytes (Makino 1999). It is assumed that it is related to modification of gene expression caused by the demethylation effect (Choi 2004). In the trial for differentiation here in this study, the cells changed morphologically upon the treatment of the 5-Azacytidine. They became more elongated, aligned parallel to each other and there was some sort of periodic grouping of the cells perpendicular to the alignment of the cells. There was also alignment of the actin fibers inside the cells parallel to the elongation direction of the cells. Induced cells showed expression of two myogenic markers that were not detected in the undifferentiated ones which indicates that there was commitment toward the myogenic lineage (Pittenger & Martin, 2004). Although there was no cell fusion or multinucleation detected commonly seen in muscle cells (Gornostaeva et al. 2006), these findings may indicate that the cells are in an initial differentiation state and that they may need continued chemical induction for complete differentiation.

The ability of the hMSCs for chondrogenic and neurogenic differentiation were also checked. For the chondrogenic differentiation, cells were grouped in pellets and induced for differentiation according to the protocol provided from Lonza (the media company). Sections from the pellets showed positive expression of collagen-II protein which is the basic protein for articular cartilage and hyaline cartilage. Cartilage of the body is made up
of about 50% of this collagen type. Detection of collagen-II confirms that the cells were
differentiated to the chondrogenic lineage and started producing the collagen-II in its
ECM that is required for the formation of the cartilage tissue (Mackay et al. 1998).

For the neurogenic lineage, a protocol from a study by Hermann et al. (2006) was
followed. The differentiation protocol involved two-step: 1) initial conversion of hMSCs
into immature neural stem cell-like cells and 2) induction of neural stem cells into glial-
like cells. In the first step, neurospheres were successfully grown and then induced to
glial cells by the supplementation of the growth factor rh-PDGF-BB in the culture media.
Control and differentiated cells were tested for the expression of two specific glial
markers (CNPase and GFAP) that both showed positive expression which was reported
before in other studies (Blondheim et al. 2006, Hermann et al. 2006). But in our trial
here, a different pattern of distribution of the CNPase enzyme was detected inside the
cells between the control and differentiated ones. In the differentiated cells the enzyme
appeared to be more distributed all over the body of the cells which may indicate some
kind of activation of its function or increase of its concentration as it is reported that it
has a role in tubulin polymerization and linking it to the cellular membranes, and its
association with cytoplasmic microtubule distribution (Lee et al. 2005, Bifulco et al.
2002).
5.1. Introduction:

The sol-gel method enables the production of porous inorganic glass under biologically benign conditions. When the liquid sol from the sol-gel process is dip-coated onto a solid substrate, a thin surface coating can be formed. This coating can be tailored with high degree of both chemical and biological activities. Molecules such as peptides can be incorporated into the gel matrix physically or chemically, creating a bioactive substrate with a desired biological effect.

In this study, I used thin film bioactive glass samples with different combinations of peptides to determine if the peptides had an effect on adherent hMSCs. Specifically, the study investigates the potential of peptide-modified bioactive glass thin films to promote cellular adhesion and osteogenic differentiation. hMSCs were used for their ability shown for differentiation to bone cells and for their importance in the field of tissue engineering. The ability to modulate the fate of hMSCs with just the interaction with the substrate give insights for the application of this functionalized bioactive glass thin films for implant coatings for better integration.

5.2. Materials and Methods:

5.2.1. Cell Culture on Materials:

Cells were prepared for the study by culturing on 10 cm tissue culture plate in hMSCs specific media (Lonza, USA) and maintained in their undifferentiated state until
they were transferred to the experimental surfaces. Cells were passaged at 80% confluency and tracked daily for any morphological changes consistent with repeated passaging and loss of stem-cell characteristics (Bourin et al. 2008).

To examine the potential of the four sets of materials (Table 4.1) prepared earlier to support the osteogenic differentiation, materials samples were placed in 48 wells plates, UV sterilized overnight and then cells were seeded at a density of 10,000 cells/sample. Plates were incubated in 5% CO$_2$ at 37°C in a humidified atmosphere and media was changed twice a week. The positive control samples were maintained in osteogenic differentiation medium specified for hMSCs from Lonza, while the experimental samples and the negative control were maintained in standard maintenance medium, to assess the influence of the substrate in the absence of soluble differentiation cues.

Table 5.1: The experimental setup for the 2D thin films *in vitro* investigation.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Experimental Samples</th>
<th>Media Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>Sol Gel (SG) Only</td>
<td>Regular Media</td>
</tr>
<tr>
<td>RGD</td>
<td>SG + RGD</td>
<td></td>
</tr>
<tr>
<td>CGG</td>
<td>SG + RGD + CGG</td>
<td></td>
</tr>
<tr>
<td>CKI</td>
<td>SG + RGD + CGG + CKI</td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>Tissue Culture Treated Cover Slips</td>
<td></td>
</tr>
<tr>
<td>Positive Control</td>
<td>Tissue Culture Treated Cover Slips</td>
<td>Differentiation Media</td>
</tr>
</tbody>
</table>
5.2.2. Immunocytochemistry:

Cells on the samples were fixed on the 2nd, 3rd, 4th and 5th weeks in 10% buffered formalin (4% paraformaldehyde) solution. After cell fixation, the samples were treated with 100% methanol, followed by permeabilization with 0.1% Triton, and blocking for non-specific binding with 10% FBS. Samples were reacted with primary antibodies for osteopontin (MPIIIB10 (1)) (Developmental Studies Hybridoma Bank, University of Iowa (DSHB)), osteonectin (AON-1) (DSHB) and osteocalcin (R&D, Minneapolis, MN) proteins. After incubation overnight at 4 °C, osteopontin and osteonectin samples were treated with Alexa Fluor 549 rabbit anti-mouse IgG1 secondary antibodies. The osteocalcin primary antibody was already conjugated. Nuclei were counterstained with Hoechst dye 33258 (Acros Organics). Fluorescent microscopy was conducted on a Zeiss Observer Z1.

5.3. Results:

5.3.1. Cell Culture:

hMSCs did adhere to the sol-gel thin films but in very small numbers as can be seen in the phase contrast images (Figure: 5.1). Only few number of cells were seen per field of view, while on the positive and negative control (no sol gel film), the cells adhered normally with the approximately the seeding density 10000 cells/cm² and proliferated as expected similar to the proliferation in the polystyrene culture dishes.
Figure 5.1: Phase contrast images showing hMSCs adhering to the experimental peptide sol gel thin films at day 2 after seeding.

5.3.2: Immunocytochemistry:

The expression of three different osteogenic proteins (osteopontin, osteonectin, osteocalcin) representing three stages of differentiation (early, middle, late respectively) was analyzed by immunofluorescent imaging giving qualitative assessment of the stage of differentiation of the cells on the different groups of samples. At the second week (first time point), cells grown on the CGG (Figure 5.2) and CKI samples showed expression of the 3 markers, while cells on the positive control showed expression of the osteopontin and osteonectin with very low expression of the osteocalcin, representing a middle stage of differentiation (Taira et al. 2003, Sun et al. 2008).

At the 3rd week, the expression of the three proteins was detected by both the experimental and positive control samples (Figure 5.3). Due to the higher number of cells that survived the seeding on the positive control samples versus the experimental samples, grouping of the cells and their organization in a trabecular bone like pattern
shapes was seen on the positive control samples. While on the experimental samples the cells remained separated and unorganized.

By the 4th and 5th weeks, no cells were found on the experimental samples while on the control samples cells were growing normally. The organization of the cells on the positive control glass coverslips became more defined and nodules of mineralization started to appear. Cells on the negative control group were comparable to the cells growing on the polystyrene culture dishes, showing no organization or mineralization nodules like the positive control group.
Figure 5.2: Immunofluorescent images of three different osteogenic protein markers at week 2 of CGG and positive control samples.
Figure 5.3: Immunofluorescent images of three different osteogenic protein markers at week 3 of RGD and positive control samples
5.4. Discussion:

In recent years, a new generation of materials with increased bioactivity has emerged. They are based on mimicking the body’s own mechanisms in directing cells fate and function. The goal of these new materials is to improve the regeneration of the body’s own natural tissues (Narayan 2010). These materials work through the release of bioactive molecules such as growth factors or have them built in their structure to trigger specific cellular reactions (Barrère 2008). Surface coating of implants with bioactive films that have this property is gaining a lot of interest in the field of tissue engineering, especially when the material to be implanted has an inferior biocompatibility such as metal implants or when specific interaction along the tissue-biomaterial interface is desired (Singh et al. 2008). A bioinert material can be turned to bioactive one by the application of a bioactive coating on its surface and greatly changing its interaction with the physiologic environment in its site of application (Shin et al. 2003).

The sol-gel technique was used to fabricate thin films coating of bioactive glass with the incorporation of 3 peptides that contain domain sequences to promote adhesion and osteogenic differentiation. The sol-gel technique was chosen because it offers the feasibility to add the peptides (as the chemical cues) during the preparation steps to be incorporated in the composition of the bioactive glass. In addition to the ability to create thin films coating of the bioactive glass through simple coating method during the sol stage of the material. Although this technique to prepare bioactive glass substrate in wet form was not reported before for our knowledge, silica sol-gel peptide ormosil thin films were used by Jedlicka et al. (2007) to promote the differentiation and growth of neurons.
Our data from examining the interaction of hMSCs grown on the wet gel thin films bioactive glass with engrafted peptides shows that the hMSCs interacted poorly to the sol-gel bioactive glass thin films and only few number of cells were able to adhere and survive up to the 3rd week on the films. This poor interaction was unclear. It could be attributed to unreacted species in the sol-gel films that are left over from the chemicals used in the preparation. Cells on the control samples (uncoated coverslips) adhered well and showed normal behavior compared to the culture dishes. All cells on all the samples were examined for the expression of 3 proteins that are expressed during the osteogenesis process (Malaval et al 1999, Frank et al 2002). These proteins represent different stages of the differentiation process, osteopontin and osteonectin are early markers that are expressed in the beginning of the osteogenic process, while osteocalcin is a late marker that is expressed in late stages and related to the mineralization of the bone tissue matrix (Born et al. 2009).

As mentioned in the results, by the 2nd week of the experiment (first time point), there were enhanced expression of all the osteogenic proteins by the experimental samples CGG and CKI in comparison to the positive control and specifically for the osteocalcin protein. The results for the second week indicate that the peptides had an osteoinductive effect on the cells and were able to faster the differentiation process. Osteopontin protein was detected in the negative control at low level of expression (weak expression) as compared to the experimental and positive control samples. This weak expression of osteopontin is acceptable as it seem that it is naturally expressed by the hMSCs in their undifferentiated state at low levels as was found in other study by another
group (Pittenger et al. 1999) and as cells differentiate to bone cells its level of expression increases.

By the 3rd week (second time point), no difference in the expression was detected between the experimental and the positive control samples. As this is a qualitative examination, it is hard to say that they are in the same state of differentiation, especially that the results from week 2 showed faster differentiation of the CGG and CKI samples. The only difference detected was the organization of the cells on the positive control samples in the trabecular bone like pattern. The lack of formation of this pattern on the experimental samples can be attributed to the very low number of the cells, their discrete spreading on the samples and the lacking of cell-cell contact. Cells on the negative control did not express the osteonectin and osteocalcin proteins at any time point. They also showed normal morphology and proliferation pattern as found on the polystyrene culture dishes which further indicate that the functionalized surfaces of the experimental samples had an osteoinductive effect on the cells.

Cells on the experimental samples did not survive after the 3rd week of the experiment which could also be attributed to their lack of cell-cell interaction. Cells secret a range of molecules through which they communicate with each other and regulate their behaviors. For example, cell-cell contact through Cadherin a transmembrane glycoprotein, trigger signaling pathways inside the cells that affect the cells responses (e.g. polarity, aggregation, migration, morphogenesis, phenotype). It was also found that defects in these contact proteins are related in some cases to abnormal
cellular behaviors and carcinogenesis (Wheelock & Johnson, 2003). Another example is the gap junctions between cells that through them different molecules are transferred between neighboring cells which regulates different processes inside the cells. Cells also secrete biomolecules into the surrounding environment. These molecules are received by other cells in the vicinity as a means of communication and interaction for regulating cell function. These ways of communication are important for the cells growth, migration, differentiation and even preservation of the cells phenotype (Bhatia et al. 1999). The loss of these means of communication and specially the cell-cell contact through the Cadherin proteins lead to impaired osteogenesis process (Stains & Civitelli, 2005).

Due to the very low number of cells on the first 3 weeks and their death afterward, other analysis such as: alkaline phosphatase measurement and qRT-PCR was not possible to be done. In future work, the data will need to be further confirmed by the repeating of the experiment and the addition of quantitative tests like alkaline phosphatase activity measurement and qRT-PCR to more precisely detect the level of differentiation of the cells. Also the investigation of the surface chemistry and surface charges of the thin films may reveal the cause of cell death.
Chapter 6: 3D scaffolds

6.1. Introduction:

The tissue engineering concept is based on the growing of functional tissues \textit{in vitro} to be implanted in the patient’s defect later for repair and remodeling to take place. Tissues need to be grown on three dimensional porous scaffolds to obtain a size of tissue that can be utilized to fill the defect area. In the case of bone, the body remodels new bone tissue according to the loading stresses in its local environment (Kalfas 2001). Thus in bone-tissue engineering a scaffold–tissue construct or a scaffold with chemical cues that can stimulate bone growth in the defect is necessary to restore lost bone and function (Jones et al. 2007).

Bioactive glasses show high potential as bone scaffold materials as they fulfill most of the required criteria for bone scaffolds (Hench 1998). Bioactive glasses prepared by the sol-gel technique especially in the composition 70\%SiO_2-30\%CaO (mol \%) were found to have the highest bioactivity among the other compositions (Saravanapavan et al. 2003). In addition, sol-gel offers the feasibility to introduce macro porosity in the structure of the scaffold through the use of different techniques during the sol state of the sol-gel transition.

Polymerization-induced phase separation was chosen to introduce macro-porosity to the scaffolds as it results in pores in the range required for bone engineering, easy to be introduced to the synthesis steps, give coral like interconnected pores network that is
essential for tissue in growth, nutrients exchange, blood vessels infiltration as the tissues grow (Marques et al. 2007). It has been also claimed to lead to mechanically stronger materials than those obtained by foaming, or fugitive phase burnout (Lofton et al. 2005), where very high pore volumes are required for interconnection of the (spherical) pores.

The synthesis and characterization of 3D peptide-modified bioactive glass scaffolds were described in Chapter 3. hMSCs were utilized in this study for their potential to differentiate into bone cells and their advantages for tissues repair. They were seeded on the samples and the effect of the different peptides combination on the cells was investigated through the analysis of the proliferation and viability of the cells on the scaffolds, and through the detection of the alkaline phosphatase activity of the cells to assess their differentiation status.

6.2. Materials and Methods:

6.2.1. Cell Seeding:

Under a sterile environment in a bio-safety cabinet, samples were added to sterile 48 well plates and incubated in culture media composed of DMEM/low glucose, and 15% FBS, supplemented by 1% penicillin/streptomycin (v/v) for 24 h prior to seeding. Media was then removed and the hMSCs were seeded at a density of 1000 cells/mm² by addition of a concentrated cell suspension in a drop-wise manner to ensure cell loading directly onto the samples. Samples were incubated at 37°C for 30 min to allow for cell attachment before new media was added to flood the samples. Samples were cultured for six time points (2, 7, 14, 21, 28 and 35 days) on the scaffolds at 37°C in a 5% CO₂
environment. Media was changed twice a week. Control samples without peptides were cultured in differentiation media composed of DMEM/low glucose, 10% FBS, 0.1 μM Dexamethasone, 10 mM β-glycerol phosphate, 0.05 mM Ascorbate and 1% penicillin/streptomycin or regular media composed of DMEM/low glucose, 15% FBS and 1% penicillin/streptomycin as positive and negative control samples respectively. The experimental setup is presented in Table 6.1.

Table 6.1: The experimental setup for the 3D scaffolds for *in vitro* investigation.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Experimental Samples</th>
<th>Media used</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD</td>
<td>Bioactive Glass + RGD</td>
<td></td>
</tr>
<tr>
<td>CGG</td>
<td>Bioactive Glass + RGD + CGG</td>
<td>Regular medium</td>
</tr>
<tr>
<td>CKI</td>
<td>Bioactive Glass + RGD + CGG + CKI</td>
<td></td>
</tr>
<tr>
<td>BIO</td>
<td>Bioactive Glass Only (Negative Control)</td>
<td></td>
</tr>
<tr>
<td>Diff</td>
<td>Bioactive Glass Only (Positive Control)</td>
<td>Differentiation medium</td>
</tr>
</tbody>
</table>

6.2.2. Scanning Electron Microscopy:

One sample from each group at each time point was fixed in 3% glutaraldehyde overnight at 4°C. Cells were dehydrated through a series of increasing concentrations of ethanol and dried using hexamethyldisilazane (HMDS, Sigma). Scanning electron microscopy was conducted on a *Philips XL30 ESEM*, using accelerating voltage of 15 kV in a low vacuum mode to scan the surface of the samples. Samples were scanned without any prior preparation.
6.2.3. Cell Proliferation:

Three samples from each group were used to assess cells proliferation on them. Samples with cell seeded on them were cultured in a phenol free media with same constitutions as described before for the normal media. At each time point 10 % Alamar Blue (resazurin) was added to the media and incubated for 18 hours at 37°C in 5% CO₂. 100 µl of media was then aspirated twice from each sample well and added to two wells in 96 well plate. The negative control was Alamar Blue added to the medium without cells. In Multiscan Plate Reader absorbance was read at 590 and 620 nm.

The number of viable cells correlates with the magnitude of dye reduction and is expressed as percentage of AB reduction. The calculation of the percentage of AB reduction (%AB reduction) is as follows according to the manufacturer’s protocol:

\[ AR_{590} = A_{590} - (A_{620} \times R_0) \]

Where \( A_{590} \) and \( A_{620} \) are the absorption at 590 and 620 nm respectively, and \( R_0 \) is a correction factor calculated from absorption of negative control.

6.2.4. Alkaline Phosphatase Activity Measurement:

A colorimetric StemTAG™ Alkaline Phosphatase Activity Assay Kit from Cell Biolabs (San Diego, CA) was used to measure the alkaline phosphatase activity of the cells growing on the samples. 3 samples from each group were collected in a 1.5 ml eppendorf tube and 300 µl of a lysis buffer (supplied with the kit) were added. Then the samples were crushed by the aid of clean spatula while in the lysis buffer to help in lysis the cells growing inside the pores of the samples. Tubes were then centrifuged at 12,000
X g for 10 minutes. Supernatant was saved as the cell lysate and stored at -80 °C until used. At the end of the experiment the alkaline phosphatase activity was measured for all samples according to the kit protocol. Briefly, 50 µl from the lysate of each sample was added to 96 well plate in triplicate and 50 µl of StemTAG™ AP Activity Assay Substrate was added to each well. Plate was incubated for 30 minutes at 37°C. After the incubation period the reaction was stopped by adding 50 µl of 1X stop solution to each well. Absorbance of each well was then read at 405 nm in a Multiscan Plate Reader. Bio-Rad Protein Assay (Bio-Rad) to measure the concentration of Bovine serum albumin as a reference protein was done and results were used to normalize the alkaline phosphatase measurements.

6.3. Results:

6.3.1. Scanning Electron Microscopy:

Attachment and spreading of the hMSCs on all the specimen types was confirmed with SEM. SEM images indicated good adhesion of the cells at day 2 on all samples (Figure 6.1). Peptide grafted scaffolds showed different hydroxyapatite layer with distinct crystals formation which was clearly visible by week one. SEM images of week 3 (Figure 6.2) show cells spreading on the surface with more flat morphology. By week 4, cells showed more spreading and formation of a monolayer on the surface with more complex network of attachment (Figure 6.3).
Figure 6.1: SEM image of BIO (left) and CKI (right) sample at day 2.

Figure 6.2: SEM images of CGG (left) and RGD (right) samples at day 21.

Figure 6.3: SEM images of CKI (left) and RGD (right) samples at day 28.
6.3.2. Cells Proliferation:

Cells were assessed for proliferation and attachment to the samples by the use of Alamar Blue dye. The dye act as an oxidation-reduction (REDOX) indicator that changes color in response to chemical reduction of growth medium resulting from cell growth, and the amount of change in the color is related to the amount of cells. So by measuring the absorption of the media with the reduced dye, calculation of the number of cells on the samples can be done.

![Figure 6.4: Alamar Blue cell proliferation assay of hMSCs on bioactive glass-peptides samples. Error bars represent standard deviation of the mean for n=3 with 5% value. (No statistical analysis was performed as this assay was done once)](image)

Results (Figure 6.4) show that cells adhered to the samples and were able to proliferate on all samples showing increase in number up to day 7. While the Diff and RGD samples maintained almost the same population of cells up to day 14 for Diff samples and day 21 for RGD, the others samples showed decrease in cells number after the first week and until the 4th week. The CGG samples although had lower cell
attachment in the beginning as shown in day 2, it did follow the same trend of cell viability as the RGD samples.

6.3.3. Alkaline Phosphatase Activity:

Comparison of the alkaline phosphatase activity of the different samples (Figure 6.5), shows that cells grown on all the peptide containing samples had increased ALP activity in compared to the control BIO sample especially at the first 2 weeks, with an increase at day 2 comparable to the Diff samples (positive control). CKI sample at 2 weeks had increased ALP activity more than the other samples, although far less than the Diff sample. CGG sample showed more steady level of ALP activity up to 4 weeks and at 4 Weeks it was the only sample that had the highest ALP activity even compared to the Diff sample.

![Alkaline Phosphatase Activity](image)

Figure 6.5: Alkaline Phosphatase Activity of the hMSCs grown on the different samples at day 2, 14, 28 and 35. Error bars represent standard deviation of the mean for n=3 with 5% value. (No statistical analysis was performed as this assay was done once)
4.4: Discussion:

Grafting of peptide-silane on the surface of 3D bioactive glass scaffold was done and the attachment of the peptide to the surface was confirmed by XPS surface analysis. The XPS analysis showed the presence of the carbon and nitrogen elements on the surface of the peptide grafted samples and not on the control samples. The attachment of the peptide-silanes to the surface is expected to be covalent attachment between the silane group attached to the peptide chain and the silanol (Si-OH) groups on the surface of the materials, rather than just electrostatic interaction.

Based on a study done by Lenze et al. 2003, they reported sustained and controlled release of laminin protein from bioactive glass scaffolds foams after covalent attachment of the protein to the surface of the scaffolds, with the normal formation of the hydroxyapatite layer and retention of the bioactivity of the material. We expect that the same mechanism could happen with our scaffolds. Peptides will be released as the material dissolves. These peptides will then be present to interact with the cells attached on the surface. The rate of the release will be related to and controlled by the rate of the scaffold dissolution.

A difference in the hydroxyapatite layer crystals shape and size was detected in the SEM studies in our experiment here between the control samples and the peptide grafted samples. This could be attributed to the presence of negatively charged side groups within the amino acids of the used peptides that act as nucleation seeds. Negatively charged groups such as carboxyl and sulfate groups attract the positively charged calcium ions through electrostatic interaction and promote the growth of the hydroxyapatite
crystals (Benesch et al. 2008). Such groups are found in amino acids like cysteine, aspartic acid and glutamic acid which are present in some locations within the peptides sequences used here. In addition to these groups, silanol and phosphate groups (that form on the glass surface upon interaction with the surrounding solution) were also reported to induce the nucleation (Takeuchi et al. 2005). So it is probably an interaction of all these groups that lead to the difference in the HA crystals formation.

The SEM images also showed that the morphology of the cells on the first week was different than the successive weeks. The cells on the first week assumed a fibroblast-like (spindle) shape on all samples (Figure 6.1) and by the 3rd week they showed more spread out flat shape indicating the formation of more adhesion points (Figure 6.2). On the 4th week, more spreading of the cells on the surface can be seen with the formation of a monolayer of cells (Figure 6.3). This increase in adherence and spreading of the cells could be explained by the formation of the hydroxyapatite crystals, with stability of the surface reactions that happen due to the dissolution of the material once they are exposed to the media and adsorption of more proteins from the media that act as ligands for cells attachment (Ducheyne & Qiu, 1999).

Proliferation analysis showed an increase in the number of cells on all samples by the end of the first week in culture. Comparing the cell proliferation profile (Figure 6.6) from day 2 to day 7 (week 1), we can see that the positive control (Diff) samples had a less significant increase. That could be due to the earlier start of differentiation of the cells by the induction media used with these samples. This can be seen also by the
equivalent number of cells on the RGD and CKI samples at day 7 (week 1) with the Diff samples, although the initial number of cells that adhered to the former samples was less than the Diff samples. The RGD samples maintained almost the same number of cells up to week 3 which indicates that the RGD peptides enhanced the cell attachment. For the CKI samples, the cells increased in number from day 2 to day 7 (week 1), but decreased in number by the second week, indicating possible differentiation. This is further supported by the increase in alkaline phosphatase activity of the CKI samples (Figure 6.5).

Figure 6.6: hMSC proliferation on bioactive glass-peptides samples. Error bars represent standard deviation of the mean for n=3 with 5% value. (No statistical analysis was performed as this assay was done once)

Analysis of the proliferation graph shows that in between the 2\textsuperscript{nd} and the 3\textsuperscript{rd} weeks, the rate of decrease in the cells number on the positive control samples was faster than
the other samples. This could be due to the early or enhanced differentiation on these samples from the effect of the inductive media and that the cells started to mature faster. From week 3 to week 4 all samples deceased with the same rate. In general, RGD and CKI samples supported the attachment of more cells than the negative control all over the experiment time period. The change in the cells numbers on the CGG samples was less profound than the other samples and they were able to maintain the cells on them more than the negative control samples although they had initial lower number of cells at day 2, which could be due to less differentiation effect on the cells as seen in the less alkaline phosphatase activity they showed than the RGD and CGG samples.

Alkaline phosphatase measurements show that the peptide grafted samples had an elevated activity equivalent to the positive control and more than the negative control samples at day 2. This shows that the peptides had an effect on triggering earlier osteogenic differentiation of the cells (He et al. 2008, Dayoub et al. 2003). At day 14, the RGD and CKI samples showed increase in the alkaline phosphatase more than the negative control and the CGG samples, with special reference to the CKI samples, which show that the peptide combination on the CKI sample have more differentiation influence on the cells than the other two samples but still less than the influence of the inductive media. CGG sample at week 4 had an equivalent amount of alkaline phosphatase activity to the positive control and it was more than any of the other peptide grafted samples or the negative control. This may indicate that this peptides combination on this sample has a weaker induction effect but more steady than the others. Negative control samples were less in the alkaline phosphatase activity at all time points. The alkaline phosphatase
measurement on all samples peaked at day 14 which is consistent with other studies (Kumar et al. 2005, Shafiee et al. 2011), as it is thought to be correlated with increasing mineral deposition and maturation of the bone cells as they become Osteocytes (Jaiswal et al. 1997).

The grafting of the different peptides on the bioactive glass that are known for their function in the adhesion and osteogenic differentiation of cells, modulated the HCA formation, increased the adhesion and osteogenic differentiation of the hMSCs. Previous studies have shown that the bioactive glass has no direct effect on the hMSCs osteogenic differentiation (Reilly et al. 2007). This shows the possibility by this technique for enhancing the bioactivity of the bioactive glass through broadening its osteoinductive property to include the hMSCs. Further investigation like qRT-PCR will be required for the confirmation of these results.
Chapter 7: Conclusions

Nature has always provided inspiration for designing materials, systems and solving problems in human health. Otto Schmitt, who was a scientist and engineer, his work coined the term *biomimetics* to describe the transfer of ideas from biology to technology, which became a common strategy in the field of tissue engineering (Harkness 2004). Tissue engineering / regenerative medicine is an emerging multidisciplinary field involving biology, medicine, and engineering for the restoration, maintaining, or enhancement of tissue and organ function (Vacanti & Vacanti, 2000).

Mimicking the action of the ECM proteins can be accomplished by the synthesis of small peptide sequences that contain the functional domains from the parental protein and grafting these peptides on the material surfaces. This modification can alter the material to selectively interact with a specific cell type or induce a desired cellular response through biomolecular recognition events (Tamerler & Sarikaya, 2009). The main idea of the biomimetic surface engineering is that cell behaviors can be controlled by these peptides that mimic parts of the ECM and that surfaces modified with these peptides can induce tissue formation conforming to the cell type seeded on the material (Durrieu et al. 2004).

Based on these previous paragraphs, our aim in this study was built. Bioactive glass is a material that has high bioactivity and can induce bone formation in bone progenitor cells but studies have shown that it has no effect on hMSCs (Leach et al. 2006, Reilly et
So we hypothesized that the potentials of the bioactive glass can be broadened to include the differentiation of hMSCs by the incorporation of peptides from proteins known for their ability to induce differentiation of hMSCs into bone cells. For that, three peptides sequences that contain domains from fibronectin, BMP-2 and BMP-9 proteins that are known to promote adhesion, differentiation and osteogenesis in hMSCs were selected and synthesized. These peptides in different combinations were then incorporated in 2D and 3D forms of bioactive glass with 70%SiO$_2$-30%CaO (mol%) composition and materials were tested for their ability to differentiate the hMSCs.

In conclusion, the experiments have shown the viability of our hypothesis. Peptides can be incorporated in the composition of the bioactive glass or grafted on its surface with the retention of the bioactivity of both the peptides and the bioactive glass. This was shown by the differentiation of the hMSCs to bone cells and the normal formation of the HCA layer on the bioactive glass surface. Moreover the HCA layer formation may be enhanced through the selective use of specific amino acids in the peptides chains that can act as nucleation seeds beside the amino acids sequence that induce the adherence or the osteogenic differentiation in hMSCs. This technique can enhance the bioactivity of bioactive glass material to increase its role in bone tissue engineering.

Future work should focus on examining more peptide combinations from other proteins to achieve better adherence, proliferation and osteoinductive effects on the hMSCs. Studying of the dissolution kinetics of the bioactive glass in the presence of the peptides and release of the peptides as the material dissolve will give insight on how the
material functions. Measuring the alkaline phosphatase activity for the hMSCs on the 2D thin films and performing of qRT-PCR analysis for the proteins expressed by the hMSCs, to understand the interaction of the cells with the material and the effect of the peptides grafted on the up and down regulation of the different genes of the hMSCs.
References


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Vitae

Mohamed Ammar was born on January 15th, 1979 in Alexandria, Egypt. In 2001, he earned a bachelor degree in dental Medicine and surgery from Alexandria University, Egypt. He practiced as a general dentist in the ministry of health in Egypt until 2004. In 2005, he joined the Tissue Engineering Laboratories in the Faculty of Dentistry, Alexandria University, Egypt. As a team member, his main research was focused on the fabrication of porous bone scaffolds from bioactive glass and testing its biocompatibility through conducting in vitro and in vivo experiments. In 2009, he was accepted for graduate studies in the materials science and engineering department at Lehigh University, USA.