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## ABSTRACT

### OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS ON THIN FILM TYROSINE DERIVED POLYCARBONATES

by  
**Tamunotonye Briggs**

Mesenchymal stem cells, harvested from adult bone marrow, are promising in the field of regenerative medicine because of the vast differentiation potential into various cell lines such as: osteoblasts, chondrocytes, adipocytes, and neurons. Osteogenic differentiation of human mesenchymal stem cells (hMSC) could be an important tool in the treatment of orthopedic deficiencies such as bone defects. The extent of *in vitro* human mesenchymal stem cell growth, adhesion, motility and differentiation into osteoblasts is a function of the material surface chemistry which is mediated by protein adsorption onto the surface. A library of tyrosine derived polycarbonates allows the tailoring of material properties to suit specific cell response by varying the structure of the polymer at the pendent chain and the incorporation of PEG in the backbone. Increasing pendent chain length increases the hydrophobicity of the surface which is hypothesized to support osteogenic differentiation at a greater extent than hydrophilic surfaces. To determine the extent of osteogenic differentiation on thin films, cell morphology, cell proliferation, biochemical assays specific for osteoblasts, cytoskeletal arrangement and cell motility were assessed. The results of this study show that increasing the pendent chain length does not cause statistically significant changes in osteogenic differentiation, however the incorporation of polyethylene glycol in the polycarbonate backbone had a profound affect on cell morphology, proliferation and mineralization.

**OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM  
CELLS ON THIN FILM TYROSINE DERIVED POLYCARBONATES**

by  
**Tamunotonye Briggs**

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Master of Science in Biomedical Engineering**

**Department of Biomedical Engineering**

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**APPROVAL PAGE**

**OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM  
CELLS ON THIN FILM TYROSINE DERIVED POLYCARBONATES**

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“If you want it, you got it, you just got to believe, believe in yourself” [46]

Believe  
Lenny Kravitz



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# **CHAPTER 1**

## **BACKGROUND**

### **1.1 Bone Defects**

It is estimated that over 500,000 bone grafts are implanted into diseased and damaged bone a year [2]. This is due to metabolic disease such as diabetes, traumatic injury and bone cancers. The traditional approach to bone graft surgery is to use autografts, allografts or biomaterials. Autografts, bone which is harvested from the patient's body, usually the hip, is preferred in many cases because the lack of an immune response. However, there is morbidity associated at the site of harvest due to infection or pain. Allograft, acellular tissue harvested from a cadaver solves the issue of donor scarcity, yet the risk of disease transmission as well as the lack of bone in-growth, limits the widespread use of this product. Biomaterials, such as metals and ceramics have a long history in orthopedic applications. Both metals and ceramics have been shown to be bioinert in most cases, meaning that the extent of biological reactions to these materials is limited. Titanium alloys were chosen in the design of orthopedic implants because of their high mechanical properties such as high modulus and load bearing properties. However, high load bearing implants impose a stress shielding effect on adjacent bone and tissue which prevents proper healing of the defect. This can cause loosening of the implant and subsequent invasive surgeries.

Tissue engineering is a field that integrates biological scaffolds such as the extracellular matrix or synthetic biopolymer scaffolds with cells and/or growth factors, for the purpose of restoring the function of a non-viable or damaged tissue or organ.

Biological or synthetic scaffolds can provide spatial, chemical and mechanical cues that guide or aid in the differentiation of cells in the area of defect. The tissue engineered approach to bone graft substitutes is engineering a bone graft substitute that would support bone ingrowth, be mechanically compatible to the host bone, and be bioresorbable [27]. Metals are difficult to process into a porous scaffold. However, ceramics and polymers could be processed into scaffolds. Bruder et al. reported that human mesenchymal stem cells (hMSCs) on a fibronectin coated porous hollow cylinders of 60% hydroxyapatite/40% tricalcium phosphate (HA/TCP) implanted in the femoral gaps of Harlan Nude rats had higher bone integration, strength and stiffness values compared to cell free implants at 12 weeks [13]. However, ceramics are not ideal in treating bone defects because of their brittle mechanical behavior and lack of bioresorbability. Bioresorbable polymer scaffolds would be the ideal substrates to induce bone ingrowth while providing mechanical support through the degradation process.

The goal of regenerative biology is to restore the function of damaged tissue and organs. The transplantation of whole viable organs and tissue is an example of regenerative medicine, however, the shortage of viable organs in the various banks to accommodate the vast majority of those suffering from diseases and afflictions is a major concern. Therefore, the focus of regenerative medicine has been placed on stem cell therapy [42]. The goal of allogenic or autologous stem cell transplantation is to either repopulate cellular defects or secrete products such as extracellular matrix at the site of damage such as segmental bone defects.

Stem cells represent a vast population of undifferentiated cells that have the ability to differentiate into more specialized cells under defined or random conditions as well as have the ability to self-renew when undergoing cell divisions. There are a variety of stem cells that differ in their differentiation potential as well as site of harvest. The most common stem cells are: embryonic stem cells, hematopoietic stem cells, and mesenchymal stem cells.

### 1.2 Embryonic Stem Cells

The most controversial of all stem cells are embryonic stem cells. Embryonic stem cells have been at the forefront of the political debate in scientific research in this country since August 9<sup>th</sup> 2001, when limitations were placed on the funding of federal research to a limited number of stem cell lines. Embryonic stem cells are harvested from the inner cell mass of 5-day old blastocysts. These blastocysts are obtained from the unwanted leftovers from fertilization treatments from *in vitro* fertilization banks. Currently, there are 155 embryonic stem cell lines in the world. Of these only 78 cell lines had been approved for federal research in the United States. In order to use these cell lines for human applications, they must be devoid of animal-derived proteins, this reduces the amount of cell lines to 22, for possible allogenic transplantation.

Embryonic stem cells are pluripotent, in that they can differentiate into cells that form the three germ layers: ectoderm, mesoderm and endoderm [35]. Embryonic stem cells have the potential for differentiating into liver, skeletal muscle, endothelial cells, chondrocytes, cardiomyocytes, hematopoietic cells, neurons, pancreatic endocrine cells and adipocytes. Despite the vast potential of these stem cells in research and possibly in

the treatment of diseases, there are many concerns that have limited the use of embryonic stem cells in tissue engineering applications. One of the concerns is the formation of teratomas, a specific tumor which contains well-differentiated cells from the three different germ layers at the site of implantation. Another concern of utilizing embryonic stem cells is its immunoreactivity. Embryonic stem cells express the cell surface marker: MHC Class I, which can be recognized by the host T cells and elicit an immune response. In order to limit the risk of an immune response, a match between the host MHC Class I and the donor embryonic cells must be made prior to implantation. This may pose a limitation in the clinical setting, unless an embryonic stem cell bank is created containing all the possible MHC Class I moieties. The current state of the public policy which limits the funding of federal research on embryonic stem cells to only 22 cell lines, would limit this endeavor. Public policy aside, the ethical debate on utilizing embryonic stem cells for research does not seem to be heading in a definitive direction.

### **1.3 Hematopoietic Stem Cells**

There are two major classes of stem cells derived from adult tissue: hematopoietic stem cells and mesenchymal stem cells. Hematopoietic stem cells (HSCs) reside in adult bone marrow close to the endosteum. Under either stochastic or deterministic conditions, hematopoietic stem cells differentiate into the various blood cells such as neutrophils, monocytes/macrophages, basophils, eosinophils, erythrocytes, platelets, mast cells, dendritic cells, B and T lymphocytes[9]. The process of hematopoiesis is tightly regulated and when disrupted can lead to severe pathologies such as lymphoma, chronic or acute leukemia. The differential potential of the hematopoietic stem cells is dependent on its

niche, which is within the bone marrow [38]. The hematopoietic stem cell niche is supported by other cells such as mesenchymal stem cells, stromal cells and osteoblasts.

Hematopoietic stem cell transplantation is the oldest and well-characterized example of stem cell transplantation, being practiced in medicine for over fifty years. This procedure is done to treat those afflicted by blood disorders such as lymphoma and leukemia. However, in order to assure successful cell engraftment, the host cell and donor cells must have an immunogenic match through the expression of histocompatibility locus antigen (HLA). The mismatch will lead to graft versus host disease(GVHD), a reaction that has severe complications and may compromise the healing process of the transplant. The *in vitro* manipulation of HSCs for tissue engineering purposes is not very successful because HSCs do not expand under *in vitro* conditions and are non-adherent. The plasticity of HSCs is limited to blood and immune cells, however, there have been a few successful attempts of differentiating HSCs to other cell types, such as hepatocytes[3]. Therefore, for the applications of tissue engineering, HSCs are not preferred.

#### **1.4 Mesenchymal Stem Cells**

Mesenchymal stem cells (MSCs) are multipotent cells harvested from adult bone marrow. MSCs have a low frequency in the bone marrow, representing only 1 out of 100,000 nucleated cells. MSCs are promising in regenerative medicine because of the vast differentiation potential into cells comprising connective tissue. Ethically, mesenchymal stem cells preferred in research because they are harvested from adults rather than the embryonic blastocyst, which is the source of debate with embryonic stem cell research.

These cells are also preferred in research because of the ease of *in vitro* maintenance and expansion.

The differentiation potential of mesenchymal stem cells is dependent on its niche, within the stromal compartment of the bone marrow, or in the case of *in vitro* culture, the local microenvironment. Mesenchymal stem cells can differentiate into osteoblasts, chondrocytes, adipocytes, as well as the stromal cells of the bone marrow which, provide support to the hematopoietic stem cells.

The isolation and culture of human mesenchymal has been well characterized. Bone marrow aspirate is taken from the superior iliac crest of the pelvis and fractionated using a density gradient solution such as Percoll [22]. A low percentage of cells attach in the initial days of culture. Yet, 12-16 days is sufficient for reaching confluence of MSCs and depletion of non-adherent hematopoietic stem cells. Cells are cultured using Dulbecco's Modified Eagle Medium containing 10% Fetal Calf Serum. Human mesenchymal stem cells are characterized by cell surface markers: CD45-, CD105+, CD44+, CD29+, and CD31- [8]. These cell surface markers differ from those expressed on hematopoietic stem cells as well as endothelial stem cells. Mesenchymal stem cells have been isolated from sites besides bone marrow, such as the periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle and teeth[14]. The differentiation potential of mesenchymal stem cells is similar when isolated from different sites.

The clinical implications of mesenchymal stem cells in tissue engineering are more promising compared to embryonic stem cells. Embryonic stem cells have the capacity of forming teratomas *in vivo*, which is not observed with mesenchymal stem cell transplantation. The immune properties of MSCs are impressive in that there is a

decreased immune response of the host to donor MSCs because they do not express MHC Class II, which is expressed on embryonic stem cells [33]. This eliminates the need to match donor MSCs to host MSCs, thereby eliminating the onset of GVHD.

The *in vitro* differentiation of human mesenchymal stem cells to specific cell lines is dependent on the supplementation of components in the culture medium as well as other manipulations such as genetic engineering. MSC differentiation into specific cell line leads to a change in cell morphology and a change in function. Chondrogenic differentiation is best achieved in a three-dimensional culture condition. The *in vitro* conditions requires: serum free medium and transforming growth factor- $\beta$  (TGF- $\beta$ ). In chondrogenic differentiation pathway, the MSCs express and release extracellular matrix components such as glycosaminoglycans. Adipogenic differentiation is achieved through the addition of isobutylmethylxanthine in the culture medium. The morphology of MSCs following adipogenic differentiation is characterized by the inclusion of large lipid vacuoles[31]. The neurogenic differentiation of MSCs is followed by the addition of isobutyl methylxanthine and dibutylryl cyclic AMP as well as brain derived neurotrophic factor and EGF.

The potential therapeutic value of MSC transplantation is promising in areas such as leukemia treatment, lung fibrosis, and muscular dystrophy. However, the therapeutic implications of mesenchymal stem cells transplantation have already been most promising in orthopedic applications. This is due to the ease of cellular engraftment into skeletal tissue defects. Mesenchymal stem cells have been transplanted to repair spine fusion, segmental bone and craniofacial defects and articular cartilage and tendon.



### 1.4.1 Osteogenic Differentiation of Mesenchymal Stem Cells

The potential orthopedic applications of MSC transplantation highlight the need to implement *in vitro* osteogenic differentiation protocols that provide a reliable, consistent culture system that will inevitably be translated into a clinical setting. Osteogenic differentiation of MSCs has been optimized under many different culture conditions. *In vitro* osteogenic differentiation can be achieved through chemical cues from defined medium, substrate cues from extracellular matrix, genetic cues and mechanical cues[23]. The presence of animal or human recombinant proteins in culture medium is a concern in osteogenic differentiation of MSCs that may be used in *in vivo* transplantation. However, the growth of MSCs is best achieved in cultures containing 10% Fetal Calf Serum and comparable serum free substitutes have not been as successful in promoting MSC proliferation. Recombinant bone morphogenic protein, (BMP) which is a member of the transforming growth factor (TGF- $\beta$ ) has been shown to induce osteogenic differentiation *in vitro*. Although the use of recombinant proteins such as BMP has been successful in MSC differentiation into osteoblasts, it is not preferred because of the delicate nature of proteins, which may denature over time, among other problems encountered with protein modification such as glycosylation. Thus, recombinant protein supplementation is not preferred in long-term cultures. Moreover, the various sources of recombinant proteins may elicit different responses from cells. This poses a problem if there is an attempt to synchronize cultures. Therefore, non-protein supplementation to serum based medium is preferred. Supplements that support osteogenic differentiation are: prostaglandins E2, Vitamin D3, L-ascorbic acid, dexamethasone and  $\beta$ -glycerophosphate. However, the most effective combination in inducing osteogenic differentiation is L-ascorbic acid,

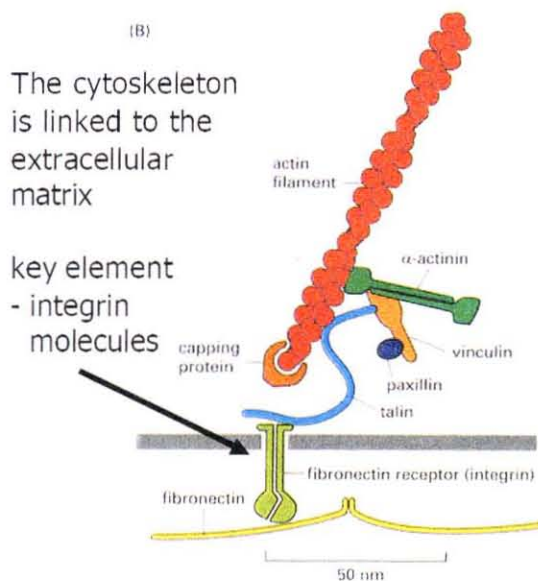
dexamethasone which is a glucocorticoid, and  $\beta$ -glycerophosphate. Vitamin D3 is a potent supplement to induce mineralization.

When added to control culture medium, these supplements elicit a specific osteogenic differentiation pathway which can be determined by observing the morphology and quantifying osteoblast biomarkers. The morphology of an osteoblast is cuboidal[12], in contrast to the fibroblast-like morphology of a mesenchymal stem cell. The change in morphology can be observed as early as two days post-osteogenic induction, however, it is more apparent four days post-osteogenic induction. Osteoblasts are characterized by the function of secreting proteins such as collagen, and mineral such as calcium into the extracellular matrix. Osteoblasts are measured for specific biomarkers such as alkaline phosphatase, an enzyme that is important in bone mineralization, calcium and osteocalcin, the most abundant non-collagenous protein in bone. *In vitro*, the presence of osteocalcin is attributed to the addition of Vitamin D3 in the culture medium[25].

Mesenchymal stem cells are rather unique from other stem cells in that they have an affinity to adhere onto tissue culture substrates. Unlike, hematopoietic stem cells which are non-adherent, mesenchymal stem cell growth, attachment and differentiation are a function of the substrate. This property can be manipulated to enhance optimum cell response *in vitro*. *In vivo*, this property is preserved through MSCs interaction with the extracellular matrix. The extracellular matrix of cartilage is comprised of proteins such as collagen, elastin and glycosaminoglycans. The extracellular matrix of bone is unique in that it is comprised of a protein and mineral component. The majority of the protein component of the bone's extracellular matrix is type I collagen. Other proteins present are

fibronectin, osteocalcin, osteopontin, bone sialoprotein[7]. The mineral component of the extracellular matrix is hydroxyapatite, which is a calcium phosphate.

MSCs interact with the extracellular matrix proteins via transmembrane receptors called integrins. Integrins are incorporated with the cytoskeletal filaments within the cell such as actin, talin and paxillin which forms focal adhesion contacts with the extracellular matrix proteins[4]. Signal transduction occurs following integrin-extracellular matrix protein binding, by way of adhesion, differentiation and motility. By manipulating the composition of the ECM proteins *in vitro* and *in vivo*, the differentiation of MSCs along a specific pathway can occur[28]. Osteogenic differentiation can occur on extracellular matrix compositions containing fibronectin, vitronectin and type I collagen[36].



**Figure 1.1** Interaction between integrin and fibronectin and its relationship to cytoskeletal organization .

## 1.5 Biomaterials

The implantation of natural or synthetic materials goes as far back to 4000 years when the ancient Egyptians used linen and animal sinew to close wounds. Biomaterials

encompass the range of materials that can be implanted into the body, including: metals, ceramics and natural or synthetic polymers or a composite of these materials. Prior to the advances in materials science in the last 50 years, biomaterials were derived from natural sources such as wood, silk and animal connective tissue. In the last 50 years, true innovations in biomaterials began to be realized. Some important milestones were: Harold Ridley used poly(methyl methacrylate) for the first intraocular lens, Vorhes invented the vascular graft, Hufnagel invented the ball and cage heart valve and Charnley invented the hip implant[32].

### 1.6 Polymers

Synthetic polymers are high molecular weight materials consisting of many repeat units, called monomers which are linked together via chemical reaction. Polymers are characterized by a distribution of molecular weight averages. This is due to the uneven distribution of chains of monomer units. The structure of the polymer, whether it is a random coil or semi-crystalline structure plays a critical role in the thermodynamic properties of the polymer such as the glass transition temperature ( $T_g$ ). This is one factor that influences the processibility of the polymer. The extent of which polymers can be fabricated and processed is much greater than metals and ceramics. The ease in processing polymers into a desired shape and texture makes it an ideal candidate for creating complex structures such as scaffolds that can have features at the micro and nano scale.

Two dimensional *in vitro* cultures are instrumental for the screening of polymers intended for two-dimensional scaffold culture. For two-dimensional *in vitro* cultures, polymers are fabricated into thin films through solvent casting, spin coating, compression molding and a number of other techniques. Both solvent casting and spin coating rely on polymers in solution. The residual solvent that may exist on the surface and can cause a cytotoxic effect, this can be detected with thermogravimetric analysis (TGA). Compression molding, on the other hand, is a film fabrication technique where films are made through the application of heat and pressure through a mold. The temperature applied to the film is dependent on the polymer's  $T_g$ . These fabrication techniques may cause films to have differences in surface topography, which may aid or hinder cell adhesion as well as differences in mechanical modulus which may affect cell differentiation.

Understanding the interface between the cells and biomaterials is integral in the successful implantation of biomaterials. The interface between the biomaterial and the cell *in vitro* and *in vivo* is mediated by protein adsorption. In fact, cells do not interact directly with the polymer. Therefore, cell behavior is a response to the adsorbed protein. Protein adsorption is the process in which proteins rapidly adsorb onto the surface of an implanted material. In *in vitro* cultures, serum proteins in the medium in the form of albumin as well as other proteins adsorb onto the biomaterial surface[1]. Protein adsorption is influenced by the surface charge or surface wettability of the polymer[40]. Surface wettability is a measure of the affinity towards water. Surface wettability can be determined by contact angle measurements, which is the angle formed by a liquid on a solid surface at the three phase boundary. Surfaces which favor water are hydrophilic and

surfaces that do not favor water are hydrophobic. It has been shown that hydrophobic surfaces greater influences protein and cell attachment.

Another attractive property of some polymers in tissue engineering applications is the ability to degrade under physiologic conditions, which is a property of bioresorbable polymers. Bioresorption occurs in two steps the first step being the process of cleaving a bond through hydrolysis or an enzymatic reaction to break down a polymer into its monomer units. At this point, the molecular weight of the polymer decreases which is followed by the loss of mechanical strength and mass loss. In the second step, the broken pieces of the implant are attacked by macrophages, which are cells of the immune system that engulf or phagocytosized foreign particles[30]. This is the beginning of a foreign body response which ultimately leads to a collagenous capsulation. The extent of this response can impede the function of an implant.

Over the last forty years, there has been considerable research on bioresorbable polymers. Bioresorbable polymers are useful in other applications such as drug delivery vehicles. The most well characterized bioresorbable polymers are classified as poly( $\alpha$ -esters): poly (glycolic acid) (PGA), poly(L-lactic acid) (PLLA), and its copolymer: poly (DL-lactic co-glycolic acid) (PLGA)[41]. These particular bioresorbable polymers are favored for orthopedic applications because of their strong mechanical properties. The polymerization of PGA is initiated by the ring opening of glycolide, which results in a linear polyester molecule. PGA has a high degree of crystallinity which contributes to its very high modulus. The degradation product of PGA is glycolic acid. The polymerization of PLLA is initiated by the ring opening of lactide, a six membered ring. The L-isomer of lactide, which is abundantly found in nature, contributes to the semi-crystalline structure

and which results in a reasonably high tensile strength and high modulus which is less than PGA. PLLA is more hydrophobic than PGA due to the presence of the methyl group. When PLLA is degraded, it releases lactic acid which is then metabolically excreted. Creating a copolymer is desirable because one can tailor the properties of the copolymer by altering the composition of the two or more polymers. PLGA, a copolymer of PGA and PLLA has a structure that is not as crystalline as PGA or PLLA homopolymer. Moreover, the degradation rate of this copolymer is controlled by altering the composition ratio of PLLA and PGA. The degradation products of PLLA, PLGA and PGA which are lactic acid and glycolic acid, are natural products. However the acidic nature of these byproducts may have a deleterious effect on the surrounding cellular environment and impede healing[39]. Furthermore, the bulk degradation of these materials produces crystalline particles which are phagocytosized by macrophages through the foreign body response, which may lead to a collagenous encapsulation.

These materials have many limited mechanical and thermodynamic properties. There are other bioresorbable polymers that are utilized in clinical settings: poly( $\epsilon$ -caprolactone), poly(orthoesters), poly(anhydrides) and poly(dioxanone). These polymers have various mechanical properties and thermodynamic properties, which can be utilized for a specific application. These polymers have also been copolymerized with other bioresorbable polymer constituents to tailor a specific property.

### **1.7 Tyrosine Derived Polycarbonates**

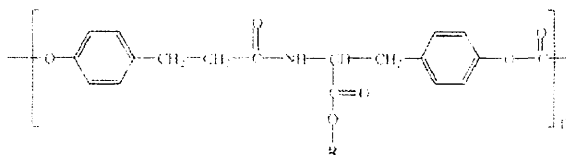
The field of bioresorbable polymers has been successful in the development of commercial medical devices and drug delivery systems for the past thirty years.

However, new approaches to the design of bioresorbable polymers with alternative degradation profiles, mechanical properties are being developed. The combinatorial chemistry approach to designing biodegradable polymers has the potential to offer an array of materials with a wide range of thermodynamic and mechanical properties. This approach developed by Dr. Joachim Kohn at Rutgers University was used to design a library of structural-related tyrosine derived polycarbonates and polyarylates, which are pseudo poly(amino acids).

In theory, poly(amino acids) would be an ideal candidate for biodegradable polymers because of the structural similarity with natural amino acids. However, poly(amino acids) were shown to have poor mechanical properties as well as elicit immunogenicity. The development of pseudo poly(amino acids) relies on synthesizing non-peptide linkages such as ester, carbonate and iminocarbonate bonds in the polymer backbone structure[10]. Tyrosine is a phenolic amino acid which when dimerized is chemically similar to Bisphenol A, a diphenol that has been used commercially to enhance the mechanical properties of polymers. Bisphenol A is not favorable in the context of biodegradable polymers because of its cytotoxicity and its inability to degrade. It was determined that rather than creating a tyrosine dipeptide, polymerizing a desaminotyrosine and desaminotyrosyl alkyl ester would provide the appropriate desired mechanical properties. Tyrosine derived polycarbonates have the same backbone but differ in the alkyl ester pendent chain. The ester bonds contribute to the degradation properties of the material. These materials are denoted by the nomenclature desaminotyrosyl-tyrosine alkyl esters (DTR) carbonate. The length of the pendent chain is denoted by the nomenclature: desaminotyrosyl-tyrosine ethyl ester (DTE),



desaminotyrosyl-tyrosine butyl ester (DTB), desaminotyrosyl-tyrosine octyl ester (DTO), and desaminotyrosyl-tyrosine dodecyl ester (DTD). In the development of tyrosine derived polyarylates, two parameters can be independently altered: the backbone and the alkyl ester pendent chain length.



**Figure 1.2** Tyrosine Derived Polycarbonate.

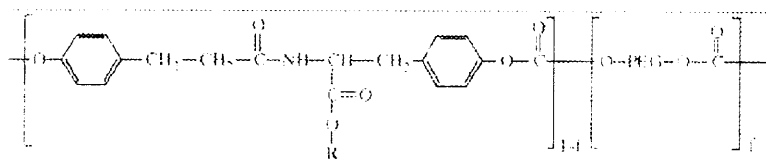
The length of the pendent chain influences the extent of most of the mechanical, thermal and surface wettability properties. This is most evident in the wide range of glass transition temperatures ( $T_g$ ) of polycarbonates which range from (52°C-93°C) [18], decreasing with the addition of each methyl group in the alkyl ester chain. The  $T_g$ 's for these amorphous materials are high enough to use under physiological conditions, where the average temperature is 37°C, and high enough to ensure reasonable thermal processing techniques. The molecular weight is also a function of the pendent chain length. The weight-average ( $M_w$ ) molecular weight of polycarbonates is between 176-450 kDa. The surface wettability decreases with the addition of methyl groups in the pendent chain, becoming more hydrophobic. The mechanical properties of these materials, measured in mechanical strength and stiffness is relatively high, making these materials ideal candidates in orthopedic applications. The rate of the degradation, which under physiologic conditions proceeds through the hydrolysis of the ester bond and the carbonate bond increases with the length of the pendent chain. The by-products of the degradation are desaminotyrosine and L-tyrosine, an amino acid which does not elicit a cytotoxic effect on surrounding cells.

The *in vivo* response to the degradation of tyrosine derived polymers compared to PLLA was evaluated in a study completed by Hooper et al. Extruded pins of poly(DTE carbonate), poly(DTE adipate) and PLLA were implanted subcutaneously in rats. The degradation of these materials was evaluated along with water uptake, mass loss and tissue response over a 570 day span. It was shown that complete resorption of poly(DTE adipate) was achieved within two years[24]. The resorption of poly(DTE carbonate) is hypothesized to take more than three years, which is similar to PLLA. The tissue response, according to the incidence of inflammation, to poly(DTE carbonate) and poly(DTE adipate) was more favorable than PLLA.

Traditionally, PEGylation, the process of poly(ethylene glycol) (PEG) incorporation into substances has been used in the pharmaceutical industry to the increase lipid clearance of highly hydrophobic drugs and proteins[21]. The incorporation of hydrophobic moieties to PEG can produce a surfactant, a material with a hydrophobic head and hydrophilic tail. This was an important discovery in drug delivery. PEG is an ideal material in drug delivery and biomedical implants because of its bioinert and non-immunogenic properties.

Surface property modification, mechanical strength and degradation rates can be affected by the incorporation of PEG through copolymerization. The properties of PEG are dependent on its molecular weight. High molecular weight PEG moieties of PEG act more as solids in contrast to liquid nature of low molecular weight moieties of PEG. The development of tyrosine derived polycarbonates provided another library of materials in which the surface properties and mechanical properties could be tailored to the application of the material. This is achieved through the manipulation of three

parameters: the percent mole fraction of PEG in the polymer backbone, average molecular weight of PEG, and pendent chain length[45]. Copolymerization is carried out by a condensation reaction of desaminotyrosyl-tyrosine alkyl ester with the desired molar fraction of PEG in the presence of phosgene. The addition of PEG has a positive correlation of water uptake and at high enough mole fraction (15%), results in hydrogel like properties. At lower PEG concentrations (less than 5%), the copolymers possess comparably strong mechanical properties such as tensile stiffness and strength, which decreases at PEG concentrations above 5% to produce flexible elastomers. The addition of PEG into the polycarbonate backbone increases the degradation rate by making it more susceptible to hydrolytic cleavage because of PEG's water uptake properties.



**Figure 1.3** Tyrosine Derived Polycarbonate PEG copolymer.

Compared to other polymers, PEG does not promote surface protein adsorption and subsequent cell attachment[17]. Protein adsorption and protein bioactivity and conformation leads to significant effects in cell adhesion and motility which can be modified through the addition of PEG in the backbone structure of polycarbonates at various molar concentrations. In a study completed by Tziampazis et al., where human fibronectin was adsorbed onto PEG polycarbonates ranging from 0-10% molar fraction, the amount of fibronectin adsorbed and its bioactivity decreased at PEG molar fractions greater than 6%[43]. Cell adhesion was also negatively affected at concentrations above 6%. Cell motility is an important parameter to study organogenesis, tissue remodeling, pathologies and bone remodeling. The incorporation of PEG in the backbone of

polycarbonates had a pronounced effect on cell motility. It was found that PEG molar concentration had a biphasic effect on cell speed, the cell speed of L929 cells increased with increasing PEG molar concentrations up to an intermediate PEG concentration (3-4%), yet at higher PEG concentrations (>8%), is motility decreased[37].

The *in vivo* and *in vitro* response to tyrosine derived polycarbonates has been well characterized. Polycarbonates have been shown to be biocompatible to a number of cell lines such as osteoblasts and fibroblasts. Because of its biocompatibility, these polycarbonates can be used in many biomaterial applications including orthopedics. Polycarbonates have been used in a number of *in vivo* orthopedic studies where mechanical strength, degradation, tissue response, bone apposition was evaluated over time. In a few studies, polycarbonates were extruded into fracture fixation pins for implantation into the transcortical femoral defect in rabbits. The polycarbonates, contained different pendent chain lengths: poly(DTE carbonate), poly(DTB carbonate), poly(DTH carbonate) and poly(DTO carbonate). In a 3-year study completed by James et al., histological samples were studied at short term and long term timepoints. It was shown that these materials were osteocompatible, meaning that there was no bone resorption and presence of inflammatory cells at the implant site[26]. However, at the bone-implant interface, bone apposition was shown to be a function of the pendent chain length, where direct bone apposition was observed at higher frequency on poly(DTE carbonate) at short term and long term timepoints compared to longer pendent chain moieties such as poly(DTB carbonate) and poly(DTO carbonate). At the bone implant interface on poly(DTB carbonate), the presence of fibrous encapsulation was observed at higher frequency (79%) than bone apposition at short term and long term timepoints. This

effect was observed with poly(DTO carbonate), where the presence of fibrous encapsulation is 83%. Whereas, the frequency of fibrous encapsulation of poly(DTE carbonate) was 27% while the frequency of bone apposition is 73%. This highly suggests that the stepwise addition of methyl groups has a dramatic affect *in vivo*.

In the canine bone chamber study completed by James et al., bone ingrowth was observed. In this study, coupons of compression molded films of poly(DTE carbonate), poly(DTH carbonate) and PLLA were placed in a chamber into a longitudinal defect in both the distal femurs of dogs. It was observed that bone ingrowth into poly(DTE carbonate) and poly(DTH carbonate) was comparable to PLLA[15]. However, the presence of fibrous encapsulation was observed at higher frequency on PLLA and to a lesser extent on poly(DTH carbonate), and not observed on poly(DTE carbonate).

The previous mentioned *in vivo* studies highlight the osteoinductive properties of tyrosine derived polycarbonates compared to standard biodegradable polymers such as PLLA. This property, along with bioresorbability and mechanical strength make tyrosine derived polycarbonates an ideal substrate for allogenic mesenchymal stem cell transplantation with the potential to differentiate into osteoblasts. Another important finding in these *in vivo* studies is the direct correlation of pendent chain length to fibrous encapsulation, a negative effect of implantation which impedes healing. Although polycarbonates have been shown to have fewer incidences of fibrous encapsulation compared to PLLA, the addition of methyl groups in the pendent chain length increases the onset of fibrous encapsulation.

## **CHAPTER 2**

### **RESEARCH OBJECTIVE**

The development of a library of polycarbonates has led to polymers with an array of material properties such as molecular weight,  $T_g$ , water uptake, and surface wettability. The goal of this study was to determine the effect of the alkyl ester pendent chain length or PEG incorporation into the polycarbonate backbone, on the osteogenic differentiation of human mesenchymal stem cells on thin film substrates. The polymers that were chosen in this study were: poly(DTE carbonate), poly(DTO carbonate), poly (DTE co 3% PEG-1K carbonate), poly(DTE co 4% PEG-1K carbonate) and poly(DTE co 5% PEG-1K carbonate). Poly(DTE carbonate) and poly(DTO carbonate) differ in the length of the alkyl ester pendent chain. The PEG copolymers have various molar percentages incorporated in the polycarbonate backbone. Two thin film preparation techniques: solvent casting and spin coating will be compared to determine their effect on osteogenic differentiation.

In this study, it was postulated that polycarbonates of lower surface wettability or higher hydrophobicity will induce adsorption of a relatively thick layer(s) of proteins that will serve as ligands for the integrin binding domains on the cell, which will lead to signal transduction by way of adhesion, osteogenic differentiation and motility in the presence of osteoinductive (OS) medium. In contrast, polycarbonates containing PEG, will have comparably higher surface wettability or higher hydrophilicity. This will induce a relatively thinner, heterogenous layer of proteins, limiting the ligand-integrin binding

and therefore, altering signal transduction by way of adhesion, osteogenic differentiation and motility. In a previous study conducted by Godbole et al.[19], hMSCs seeded on polyarylates of varying degrees of surface wettability had greater osteogenic differentiation on the more hydrophobic surfaces. Therefore, in this study it is hypothesized that the addition of methyl groups in the pendent chain, from ethyl to octyl, which results in an increase in the hydrophobicity, will have an increased effect on osteogenic differentiation. In contrast, the incorporation of PEG into the polycarbonate backbone will have a decreased effect on osteogenic differentiation due to the hydrophilicity.

*In vitro* osteogenic differentiation will be evaluated with standard quantitative biochemical marker assays, qualitative morphology and cytoskeletal observations, quantitative protein adsorption measurements and cell motility measurements.

## **CHAPTER 3**

### **EXPERIMENTAL METHODS:**

#### **3.1 Polymer Processing**

Poly(DTE carbonate), poly(DTO carbonate), poly(DTE co 3% PEG-1K carbonate), poly(DTE co 4% PEG-1K carbonate) and poly(DTE co 5% PEG-1K carbonate) were obtained from the laboratory of Dr. Joachim Kohn at Rutgers University. It was important to maintain consistency so lot numbers were maintained throughout the study as much as possible. The molecular weights were obtained from gel permeation chromatography (GPC).

Thin film substrates were fabricated using two different methods: solvent casting and spin coating. Solvent cast films were used in a 96 well plate format while spin coated films were used in a 24 well plate format.

##### **3.1.1 Solvent Casting**

A 1% (w/v) polymer solution was made in methylene chloride and filtered with a 0.45  $\mu\text{m}$  PTFE filter (Whatman). 300  $\mu\text{L}$  of polymer solution was pipetted into each well of a 96 well polypropylene plate. The plates were placed in a chemical hood overnight for polymer solvent evaporation.



### 3.1.2 Spin Coating

Spin coated disks were prepared following a protocol developed by Brocchini[11]. Glass cover slips (15 mm) were subjected to a cleaning and poly(styrene silane) coating prior to spin coating. Glass coverslips were sonicated twice in 25% NaOH for ten minutes and then twice in 25% HCl for ten minutes. The glass cover slips were sonicated in 2% Micro detergent for 30 minutes twice and then rinsed with deionised water following each sonication step. The glass cover slips were then sonicated twice in ethanol for 5 minutes and methylene chloride twice for 5 minutes. The cover slips were then twice rinsed in ethyl acetate and then sonicated in a 2.5% (w/v) poly(styrene-silane) copolymer-ethyl acetate solution and then soaked for an additional 10 minutes. The cover slips were then placed in a 60°C vacuum oven for 48 hours to anneal the poly(styrene-silane) onto the surface. Cover slips were removed from vacuum oven and cooled to room temperature then rinsed twice with ethyl acetate, methanol and ethyl acetate. The cover slips were left to air dry on aluminum prior to spin coating. A 2.5% (w/v) polymer solution in methylene chloride was made and filtered with a 0.45 µm PTFE syringe filter (Whatman). Cover slips were placed in an enclosed chamber of constant humidity (less than 20%) and temperature, on top of the spin coater (Headway, Garland, TX). Polymer solution was added to cover slip and the cover slip spun at 2000 rpm for 20 seconds. Cover slips were placed in tissue culture polystyrene (TCPS) petri dish prior to experiments. Cover slips were fixed to the bottom of TCPS 24 well plates with methylene chloride. Plates were degassed for at least 48 hours to ensure methylene chloride evaporation.

Air-water contact angle measurements were taken on spin coated disks made of a 1% polymer solution using the methods described by Brocchini. Contact angle measurements were made on a goniometer (Rame-Hart, Netcong, NJ) using double distilled waters as the probe. A sessile drop was deposited on the spin coated glass disk of approximate diameter of 2.5 mm.

### 3.2 Cell Culture

In this study, human mesenchymal stem cells (hMSCs) were isolated from commercially obtained (Cambrex) bone marrow aspirates collected from the superior iliac crest of the pelvis of male donors (18-36). The isolation method described in detail by Haynesworth, proceeds with washing the marrow sample with (Phosphate Buffered Solution) PBS then centrifuging the sample in a 70% density gradient solution at 13,000g for 20 minutes. The hMSC fraction was collected and then plated into tissue culture polystyrene flasks (Nunc) with control medium: Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% Fetal Bovine Serum (Hyclone) and 1% Antibiotic-Antimycotic (Invitrogen), then placed in a 37°C, 5% CO<sub>2</sub> containing humidified incubator. On average, confluency of hMSCs was achieved within 12-16 days. At the point of near confluency, cells were detached from the substrate with 0.25% Trypsin-EDTA (Invitrogen). Cells were resuspended in control medium and centrifuged at 900 g for 5 minutes. The cells were then collected and replated into a new tissue culture flask. This procedure called serial passaging or subculturing was done up to a maximum of 5 passages. After each passage, cells were cryopreserved in freezing medium containing 90% Fetal Bovine Serum and 10% DMSO. The cryopreserved cells were stored in a liquid nitrogen tank until the initiation of an experiment.

In this study, *in vitro* osteogenic differentiation was induced by adding osteoinductive medium (OS medium) which is control medium supplemented with 10 mM beta glycerophosphate (Sigma, St. Louis, MO), 50µM L-ascorbic acid phosphate (Wako, Richmond, VA) and 100 nM of dexamethasone (Sigma), 24 hours after initial hMSC seeding.

### **3.3 Biochemical Assay Experiment Design**

In this study, osteogenic differentiation of hMSCs was determined by observing cell morphology and quantifying cell proliferation and osteogenic biomarkers such as alkaline phosphatase, calcium. A baseline of osteogenic differentiation was determined by comparing undifferentiated hMSCs to differentiated hMSCs.

#### **3.3.1 Cell Morphology**

Cell morphology was observed at various points of the 14 day culture. Cells were observed under bright field, inverted light microscope (Nikon Eclipse TS 100) at 10X, 20X and 40X. Images were taken with a digital camera (Nikon DXM 1200F) and were visualized with an image analysis software (Metavue, Downingtown, PA).

#### **3.3.2 Cell Proliferation**

Cell proliferation was determined with the PicoGreen® ds DNA quantitation kit (Molecular Probes, Carlsbad, California). The PicoGreen reagent fluorescently labels double stranded DNA which is correlated to cell number using a standard curve. Standards of known cell number and samples were prepared by lysing cells with 0.1% Triton X-100 which were pipetted into each well of a 96 well plate. PicoGreen reagent was prepared in a 1:200 dilution in buffer solution. 100  $\mu$ L of the reagent was pipetted into standards and samples. The fluorescence was detected with FLX800 microplate reader (Biotek, Winooski, Vermont) at 480 nm excitation/ 520 nm emission.

### 3.3.3 Alkaline Phosphatase

The activity of alkaline phosphatase was determined by quantifying the conversion rate of para-nitrophenyl phosphate (p-Npp) to para-nitrophenol (p-Np) which occurs in the presence of alkaline phosphatase. Standards of p-Np were prepared in phosphatase buffer. The samples were prepared by lysing cells with 0.1% Triton X-100, and pipetting 25  $\mu$ L of the sample into a 96 well plate with 75  $\mu$ L phosphatase buffer. The samples and standards were incubated at 37°C for 30 minutes in a water bath. The reaction was stopped by adding 0.1 N NaOH into each well. The absorbance was read at 405 nm with an absorbance plate reader (Molecular Devices). The activity was normalized to cell number determined from the cell proliferation data, is expressed as nmol of p-Np/min/cell.

### 3.3.4 Calcium

The amount of calcium present in the extracellular matrix of the samples was determined using the Calcium Kit (Thermal Electron). Calcium standards (Sigma) were prepared in 0.5 N HCl. The samples were prepared by hydrolyzing the substrates in a 0.5 N HCl solution on a vortex overnight. The samples were centrifuged and the supernatant was collected. A working solution provided by the kit was prepared and added to the samples and standards. The presence of calcium was indicated by a strong purple color which was detected with an absorbance plate reader (Molecular Devices) at 570 nm. Calcium was also normalized to cell number.

### 3.4 96 Well Plate Experiments

Polymers were solvent cast onto 96 well polypropylene plates in methods detailed above. Cells grown on tissue culture polystyrene (TCPS) served as the internal control for the experiment (refer to Appendix A). Each polymer had a sample size of 4 wells ( $n=4$ ).

On the day that the experiment is initiated, day 0, cryopreserved cells were thawed and counted using Trypan Blue Exclusion dye (Sigma) and a hemacytometer. A cell suspension in control medium was prepared and 10,000 cells ( $3.1 \times 10^4$  cells/cm<sup>2</sup>) were seeded into each well. One day after initial cell seeding, osteogenic differentiation was induced with OS medium in designated wells.

Throughout the duration of the experiment (14 days), undifferentiated cells were maintained in control medium and differentiated cells were maintained in OS medium which were all fed twice a week. On day 12, osteogenic cells were cultured with medium supplemented with Vitamin D3: 10mM of  $\beta$ -glycerophosphate, 5 $\mu$ M of L-ascorbic acid phosphate and 10 nM of Vitamin D3, to induce osteocalcin production. The cells were harvested for biochemical assays at days: 4, 7, 11 and 14. At day 4, cell proliferation and alkaline phosphatase assays were conducted. On days 7, 11 and 14, cell proliferation, alkaline phosphatase and calcium assays were conducted.

### 3.5 24 Well Plate Experiment

Spin coated disks of polymers were fixed onto TCPS plates in methods detailed above. Cells grown on tissue culture polystyrene (TCPS) served as the internal control for the experiment (refer to Appendix A). Each polymer had a sample size of 3 wells ( $n=3$ ).

On the day that the experiment is initiated, day 0, cryopreserved cells were thawed and counted using Trypan Blue Exclusion dye and a hemacytometer. Due to the limited amount of cells available, a cell suspension of 40,000 cells ( $2.2 \times 10^4$  cells/cm<sup>2</sup>) were seeded into each well rather than ( $3.1 \times 10^4$  cells/cm<sup>2</sup>). Osteogenic differentiation and cell culture was similar to 96 well plate experiments. The cells were harvested at timepoints: days 7, 11 and 14 for cell proliferation, alkaline phosphatase, calcium assays.

### 3.6 Cytoskeletal Staining

Cytoskeletal organization was observed by fluorescently staining the f-actin filaments with Alexa Fluor® 488 phalloidin (Molecular Probes, Eugene, OR). Cells were seeded in the 96 well plate format. Each polymer had a sample size of 2 wells (n=2).

At day 0, cryopreserved cells were thawed and counted. A cell suspension in control medium was prepared and 10,000 cells ( $3.1 \times 10^4$  cells/cm<sup>2</sup>) were seeded into each well. 24 hours after initial cell seeding, osteogenic differentiation was induced with OS medium in designated wells. Cells were harvested for the staining at timepoints: 1 hours, 5 hours, 24 hours, day 4, day 7, day 11 and day 14. Cells were fixed with formaldehyde for 20 minutes, rinsed with PBS and fixed with 0.1% Triton X-100 for 5 minutes. The cells were then rinsed with PBS 2-3 times. The cells were then stained with a 1:100 dilution of the Alexa Fluor 488® phalloidin in PBS for a minimum of 40 minutes. The cells were then rinsed with PBS 2-3 times and fixed with anti-stain. Fluorescent images were obtained using the 450 nm-490 nm excitation/505 nm emission filter on the Nikon fluorescence microscope.

### 3.7 QCM-D

The adsorption of fetal bovine serum (FBS) on polymer surfaces was measured in real-time with a Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) instrument (Q-sense AB, Goteborg, Sweden). In the Q-CMD, an alternating electric field is applied to a quartz crystal at resonance frequencies of 5 MHz or at one of its overtones of: 15 MHz, 25 MHz, 35 MHz and 45 MHz. Protein adsorption or desorption is measured in the change in frequency ( $\Delta f$ ) and dissipation ( $\Delta D$ ) of the quartz crystal due to the increase or decrease of mass bound to the crystal. The adsorbed mass thickness and viscosity can be obtained by modeling the  $\Delta f$  and  $\Delta D$  data using the Voight model available in the Q-Sense software package.

Sample preparation is described in detail in Weber's paper (2005) [44]. Gold-coated quartz crystals (5 MHz, Q-Sense, Goteborg, Sweden) were spin coated with a 1% w/v polymer solution in methylene chloride. The polymers used for this analysis were: poly(DTE carbonate), poly(DTE co 3% PEG-1K carbonate), poly (DTE co 5% PEG-1K carbonate) and poly(DTO carbonate). The crystals were then treated in a solution containing 30%  $H_2O_2$ ,  $NH_4OH$  and deionized water for 15 minutes at 80°C. The crystals were then rinsed with deionized water, dried under nitrogen and exposed to UV and ozone for 10 minutes and rinsed with pure ethanol prior to use.

Protein adsorption experiments occurred at 37°C under a flow rate of 20  $\mu L/min$ . In one experiment, a 10% solution of FBS (Sigma) in Phosphate Buffered Saline (PBS) was adsorbed onto the polymer coated quartz disks. Protein-free PBS was added after surface saturation to determine the irreversibility of protein adsorption. The change in



frequency ( $\Delta f$ ) and dissipation ( $\Delta D$ ) was determined at the 9<sup>th</sup> overtone of 45 MHz and plotted for each polymer sample.

### 3.8 Cell Motility

The motility of hMSCs was observed and measured using confocal microscopy. On day 0 of the experiment, cryopreserved hMSCs were thawed and counted. A concentration of 3000 cells/cm<sup>2</sup> was seeded onto spin coated disks of: poly(DTE carbonate), poly(DTE co 3% PEG-1K carbonate), poly(DTE co 4% PEG-1K carbonate) and poly(DTE co 5% PEG-1K carbonate) which had been mounted inside a glass chamber. Each chamber was collected at timepoints: 18 hours, days 4, 7, 11 and 14. At day 0, 3 hours after initial cell seeding, cells were stained with 1  $\mu$ M of the fluorescent probe, Cell Tracker (Molecular Probes, Eugene, OR) in control medium for 30 minutes at 37°C. The Cell Tracker solution was replaced with CO<sub>2</sub> independent medium (Invitrogen) and the chamber was placed on a 37°C incubated microscope stage on a confocal microscope (Leica). Ten sample areas which had approximately 200 cells per area, were taken of each film. Images were taken every 10 minutes for 18 hours. Cells in the remaining chambers were induced with OS medium, 24 hours after initial cell seeding and placed in a 37° C, 5% CO<sub>2</sub> incubator until the date of harvest. On the date of harvest, the medium in the chamber was replaced with the Cell Tracker solution for 30 minutes at 37°C. The same procedure described above was followed with the remaining cell cultures to track cell motility.

The parameters that defined cell motility in this study were: average velocity, which is the rate of displacement over increments of time, random motility coefficient,

and persistence time, which is the length of time a cell travels in a straight line. These parameters are displacement dependent, where displacement was measured as a function of time, by tracking the x and y coordinates of the cell centroid, using Image Pro software (Media Cybernetics, Silver Spring, MD). The calculation of the persistence time and random motility coefficients as a function of time, was derived from the following equation:  $D^2(t_i) = 4\mu(t-P)$ , [29] where  $D^2(t_i)$  is the squared displacement as a function of time,  $\mu$  is the random motility coefficient and  $P$  is the persistence time. Average velocity,  $S$ , is obtained from the calculation:  $D^2 = 2S^2P[t-P(1-e^{-t/p})]$ . Matlab was used to compute calculations.

## CHAPTER 4

### RESULTS AND DISCUSSION

#### 4.1 Contact Angle Measurements

One characterization method of the surface is the contact angle measurement. Contact angle measurements are an indication of surface wettability, which in the case of tyrosine derived polycarbonates is modified by the addition of methyl groups in the alkyl ester pendent chain. It is observed in Table 4.1, that the average contact angle  $10^\circ$ , when going from poly(DTE carbonate) to poly(DTO carbonate), an addition of 6 methyl groups. Likewise, the incorporation of PEG into the polycarbonate structure is lower compared to poly(DTE carbonate), indicating that these polymers: poly(DTE co 4% PEG-1K carbonate) and poly(DTE co 5% PEG-1K carbonate) are more hydrophilic than poly(DTE carbonate).

**Table 4.1** Contact Angle Measurements, n=3

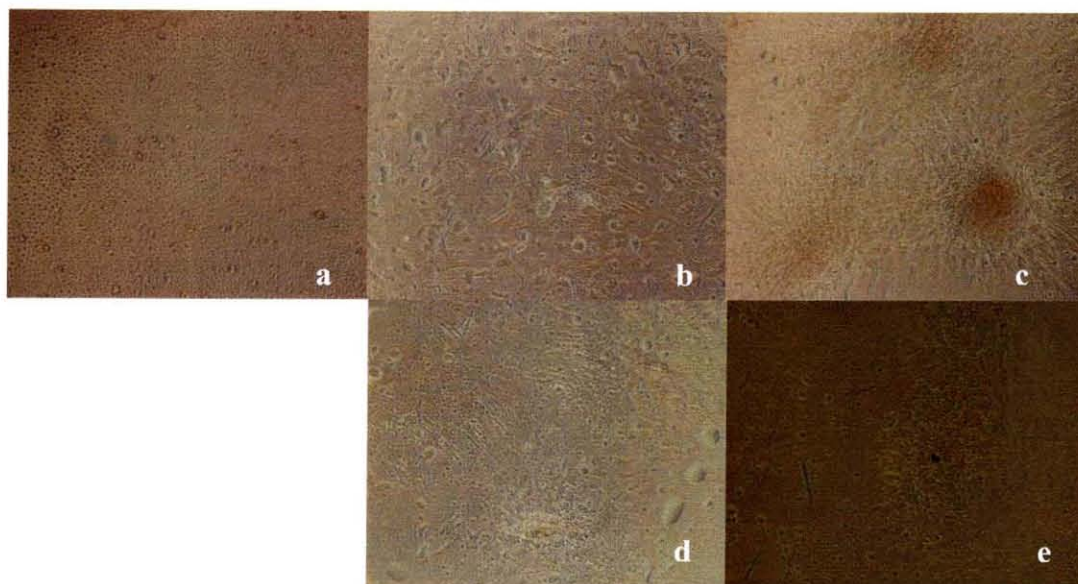
| Material                                   | Contact Angle / $^\circ$ | St. Dev. |
|--|--------------------------|----------|
| Poly(DTE carbonate)                        | 77.7                     | 0.6      |
| Poly(DTO carbonate)                        | 87.3                     | 4.6      |
| Poly(DTE-co-4%PEG <sub>1K</sub> carbonate) | 71.7                     | 2.0      |
| Poly(DTE-co-5%PEG <sub>1K</sub> carbonate) | 72.3                     | 2.5      |

## 4.2 Cell Morphology

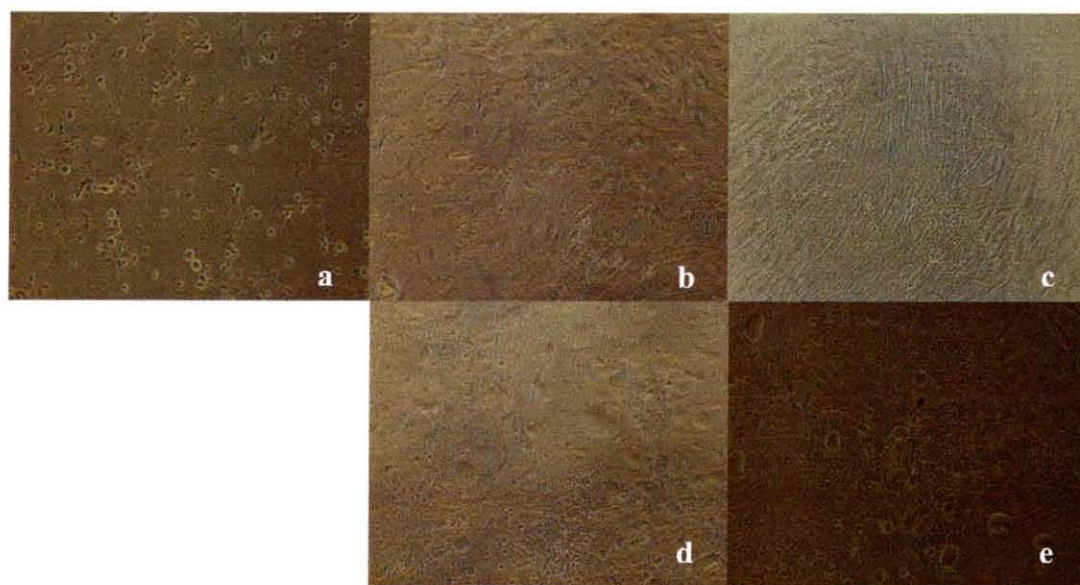
In this experiment, cell morphology is a visual representation of the cells on the substrate. Below are bright field images of undifferentiated hMSCs and osteogenic differentiated hMSCs (OS) cells at various timepoints in a 14 day culture. Experiments for both 96 well plate and 24 well plate were conducted in October 2005 using hMSC, Donor 2, passage 2. Undifferentiated hMSCs on poly(DTE carbonate), poly(DTO carbonate) (Figure 4.1c and Figure 4.2c, respectively) are characterized by a fibroblast-like morphology, while undifferentiated hMSCs on poly(DTE co 5% PEG-1K carbonate) are characterized by aggregates (Figure 4.3c). Osteogenic differentiated hMSCs (OS) are characterized by a cuboidal morphology. At high confluence, by day 7, the monolayer has a cobble-stone like morphology (Figure 4.1e and 4.2e). However, cells on poly(DTE co 5% PEG-1K carbonate) do not produce a monolayer, rather cells on poly(DTE co 5% PEG-1K carbonate) form aggregates as early as four hours after initial cell seeding (Figure 4.7a).

### 4.2.1 96 Well Plate Experiment

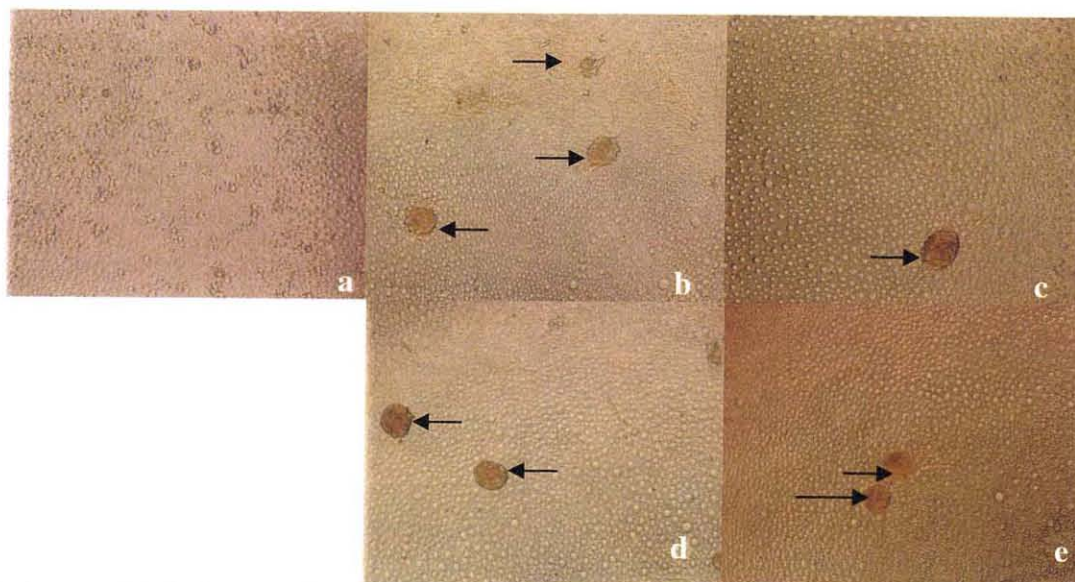
Bright-field images of cells on solvent cast films.



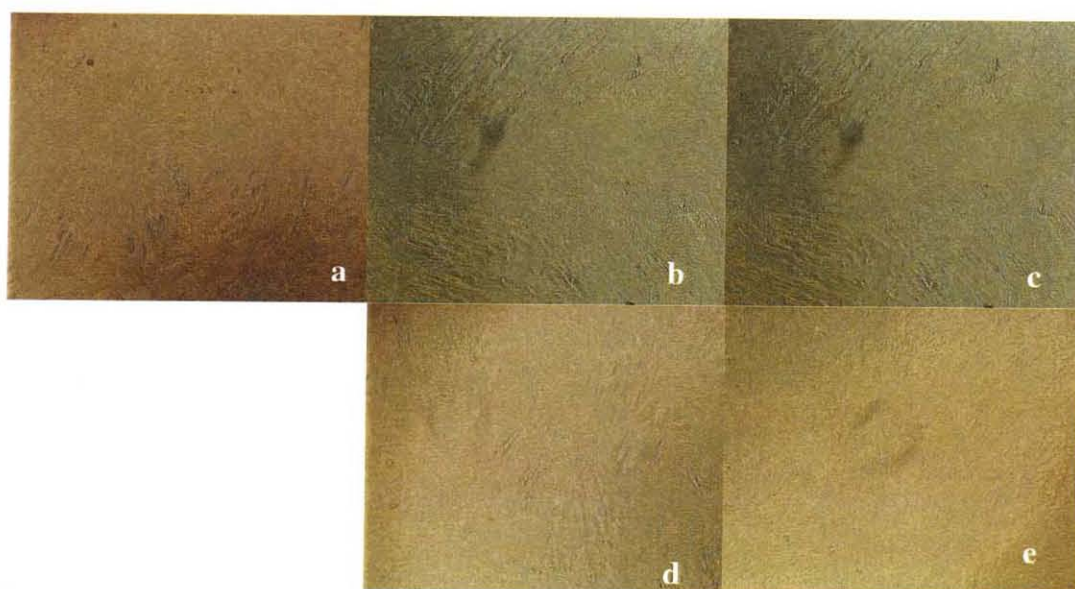
**Figure 4.1** Cells on poly(DTE carbonate), 10X: undifferentiated hMSCs at (a) 4 hours, (b) day 4, and (c) day 7; OS cells at (d) day 4 and (e) day 7.



**Figure 4.2** Cells on poly(DTO carbonate), 10X: undifferentiated hMSCs at (a) 4 hours, (b) day 4, and (c) day 7; OS cells at (d) day 4 and (e) day 7.



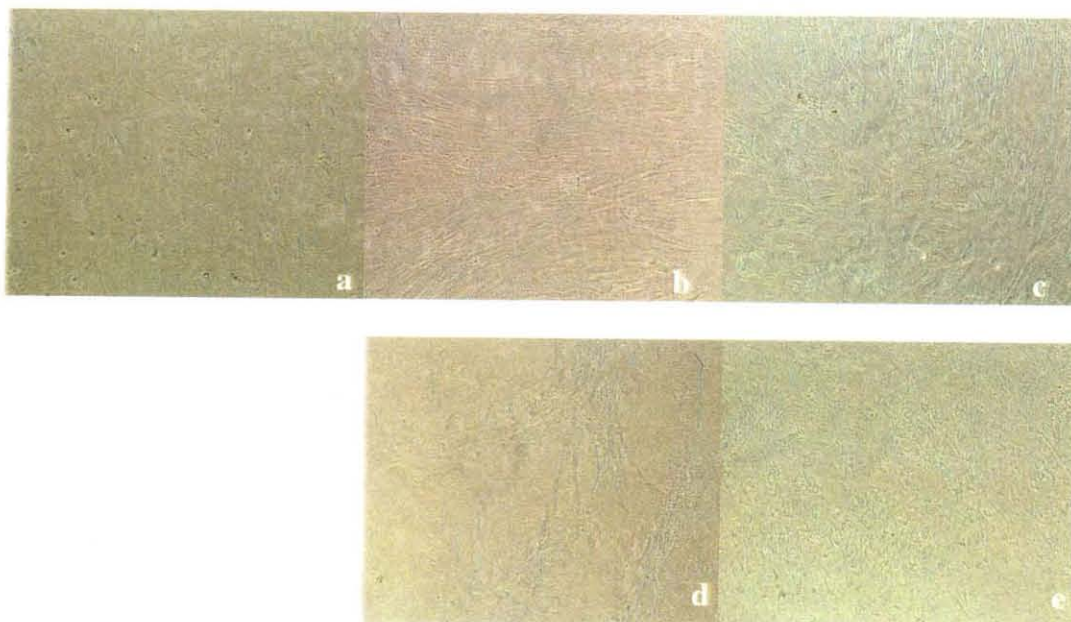
**Figure 4.3** Cells on poly(DTE co 5% PEG-1K carbonate), 10X: undifferentiated hMSCs at (a) 4 hours, (b) day 4, (c) day 7; OS cells at (d) day 4 and (e) day 7. Arrows indicate areas of cell aggregation.



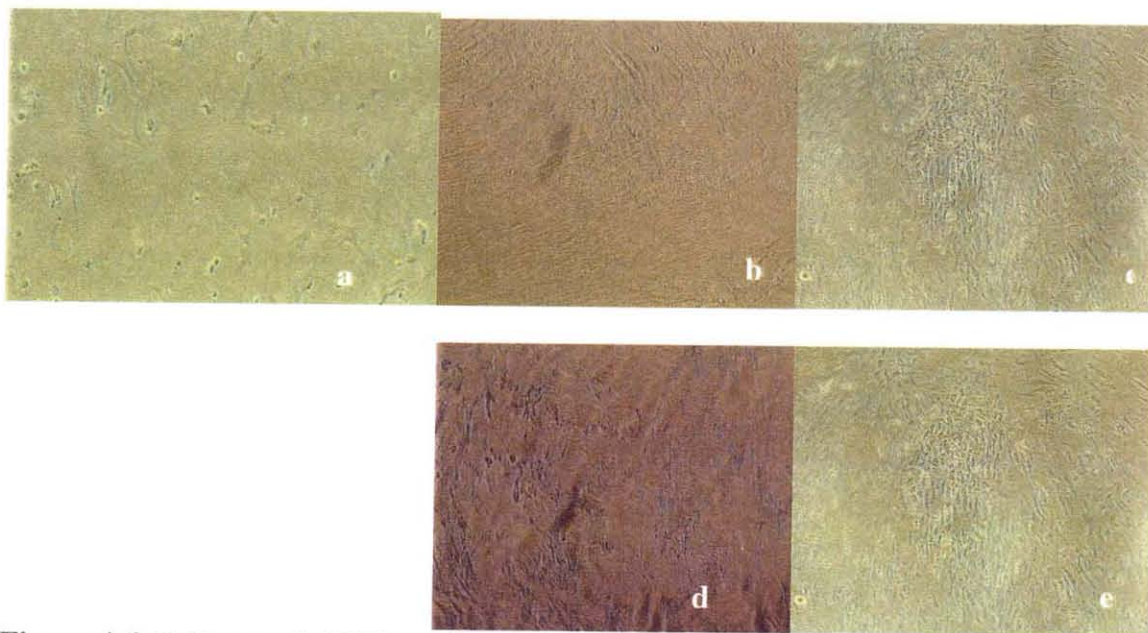
**Figure 4.4** Cells on tissue culture polystyrene (TCPS), 10X: undifferentiated hMSCs at (a) 4 hours, (b) day 4, (c) day 7; OS cells at (d) day 4 and (e) day 7.

### 4.2.2 24 Well Plate Experiment

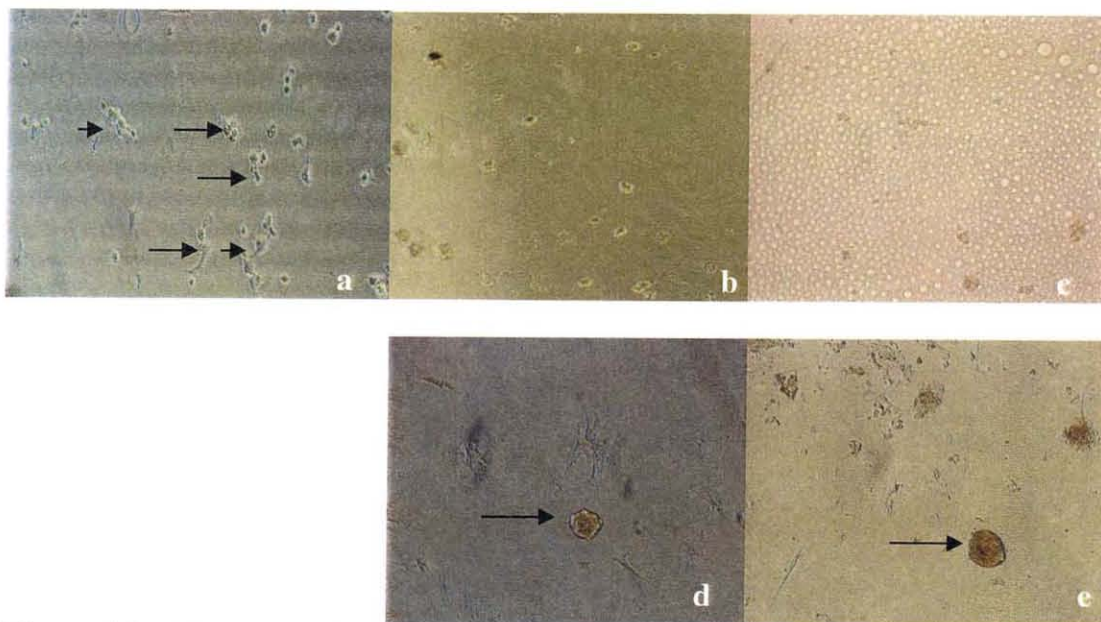
Bright-field images of cells on spin coated disks.



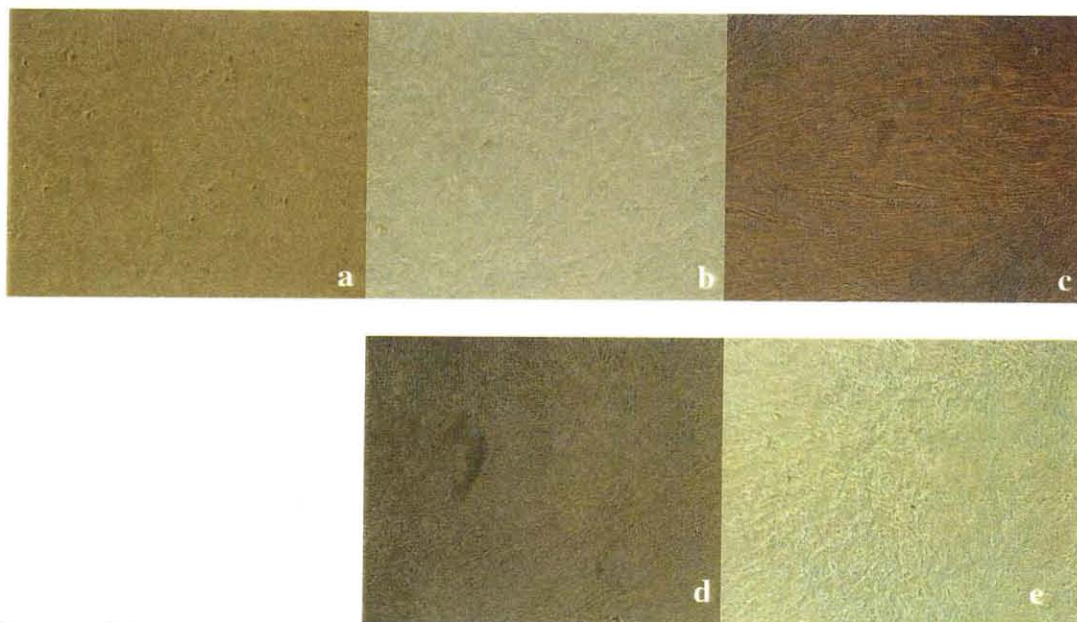
**Figure 4.5** Cells on poly(DTE carbonate), 10X: undifferentiated hMSCs at (a) 4 hours, (b) day 7 and (c) day 11; OS cells at (d) day 7 and (e) day 11.



**Figure 4.6** Cells on poly(DTO carbonate), 10X: undifferentiated hMSCs at (a) 4 hours, (b) day 7 and (c) day 11; OS cells at (d) day 7 and (e) day 11.



**Figure 4.7** Cells on poly(DTE co 5% PEG-1K carbonate), 10X: undifferentiated hMSCs at (a) 4 hours, (b) day 1 and (c) day 3; OS cells at (d) day 4 and (e) day 7. Arrows indicate areas of aggregation.



**Figure 4.8** Cells on TCPS, 10X: undifferentiated hMSCs at (a) 4 hours, (b) day 4, and (c) day 7; OS cells at (d) day 7 and (e) day 11.

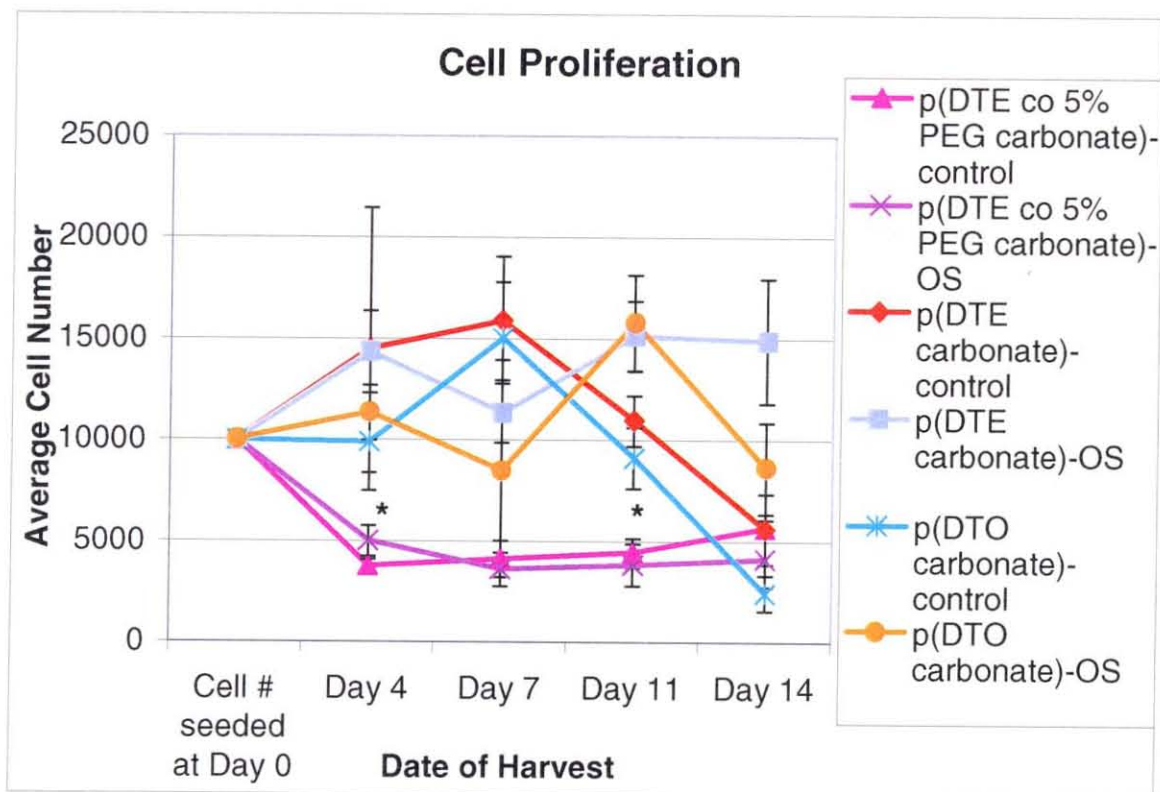


### **4.3 Biochemical Assays: 96 Well Plate Experiment**

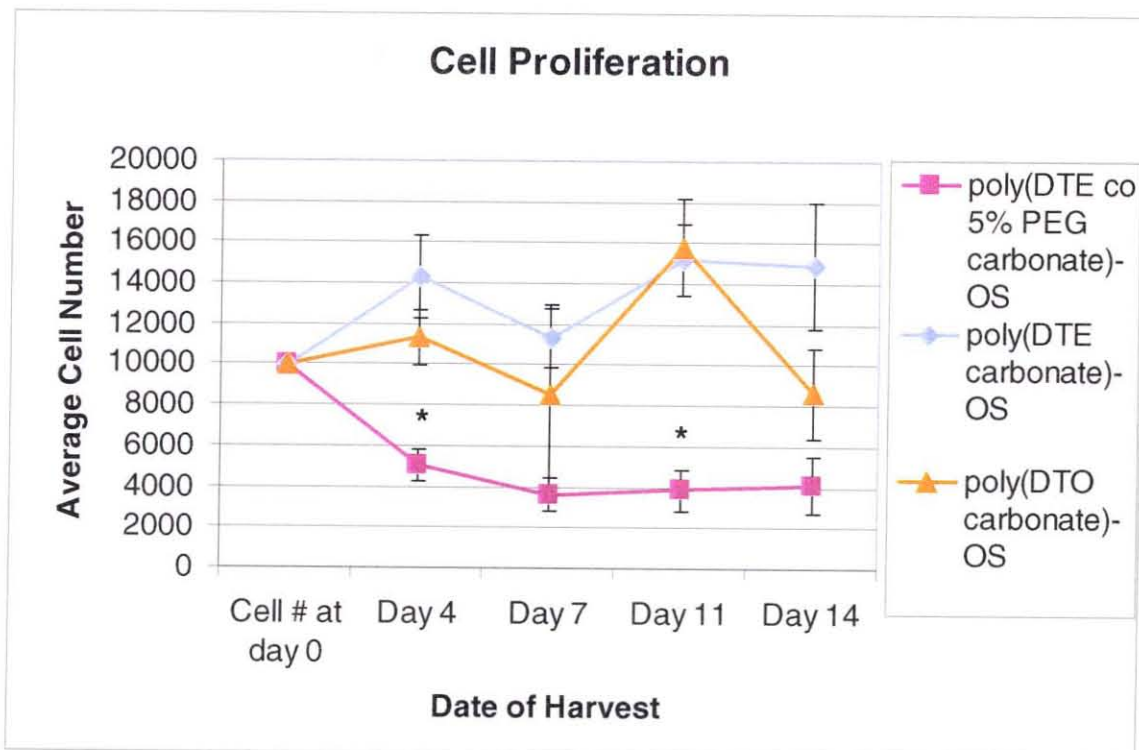
The following section is the quantitative representation of osteogenic differentiation in terms of cell proliferation, alkaline phosphatase activity and calcium production on solvent cast films in 96 well plates. Below are the results of an experiment conducted in July 2005, using hMSCs, Donor 2, passage 2. For each experiment, two graphs are represented: one representing undifferentiated hMSCs and osteogenic differentiated hMSCs (OS) (Figures 4.9, 4.11 and 4.13), and the other representing only osteogenic differentiated hMSCs (OS cells) (Figures 4.10, 4.12, 4.14 and 4.15). Data analysis was conducted using the Tukey-Kramer test between the OS cells on the polymer substrates.

#### **4.3.1 Cell Proliferation**

In this experiment cell proliferation over a 14 day culture was quantified using the DNA assay (Figure 4.9 and 4.10). For the duration of the experiment, the number of cells was much lower on poly(DTE co 5% PEG-1K carbonate) compared to the other materials. On poly(DTE carbonate) and poly(DTO carbonate) substrates, cell number reached a maximum at either days 7 and 11. At day 14, cell number sharply decreases on all polymers except for OS cells on poly(DTE carbonate). This effect could be attributed to contact inhibition of the substrate.



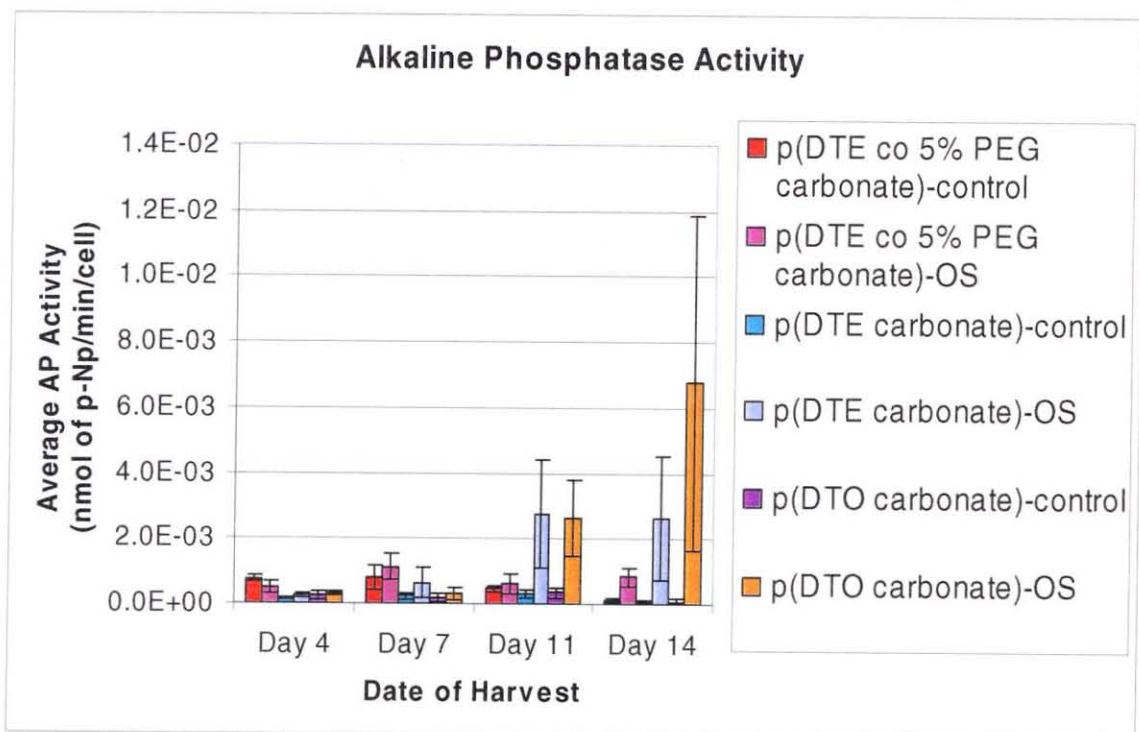
**Figure 4.9** Cell proliferation of undifferentiated hMSCs (control) and OS cells on polymer substrates. Asterisks indicate statistical difference, ( $p < 0.05$ ) between OS cells on poly(DTE co 5% PEG-1K carbonate) and OS cells on both poly(DTE carbonate) and poly(DTO carbonate).



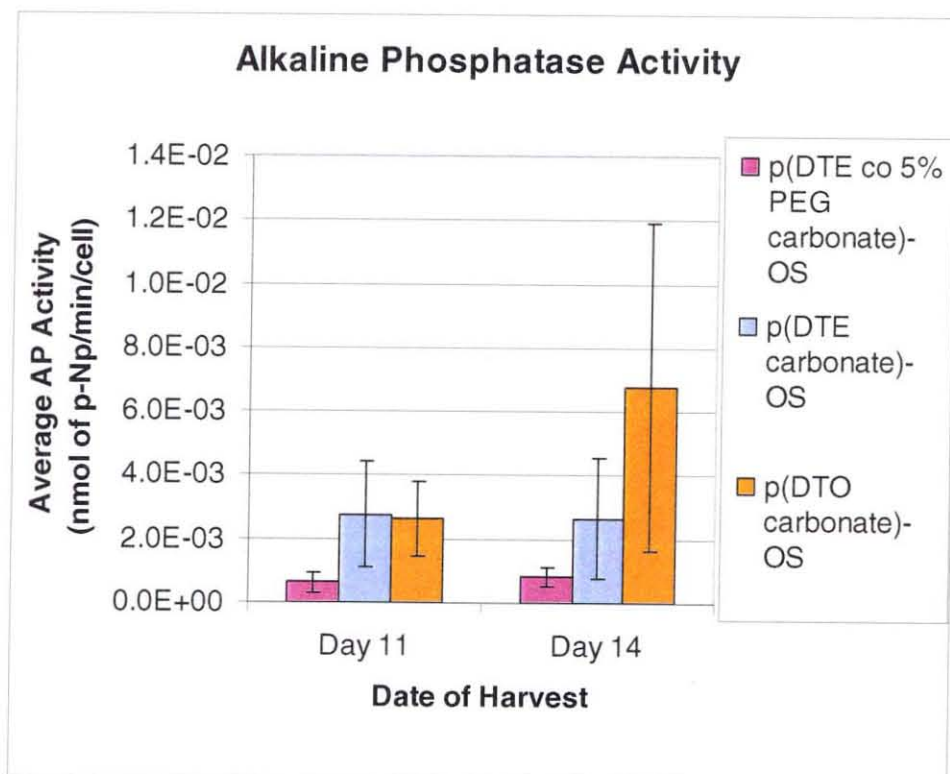
**Figure 4.10** Cell proliferation of OS cells on polymer substrates. Asterisks indicates a statistically significant difference ( $p < 0.05$ ), between poly(DTE co 5% PEG-1K carbonate) to both poly(DTE carbonate) and poly(DTO carbonate).

#### 4.3.2 Alkaline Phosphatase

Alkaline Phosphatase activity is a marker for osteogenic differentiation. Alkaline Phosphatase activity was negligible in undifferentiated hMSCs. Alkaline Phosphatase activity of OS cells wasn't apparent until after day 11 (Figure 4.11 and Figure 4.12). Alkaline Phosphatase activity in OS cells on poly(DTE co 5% PEG-1K carbonate) was much lower compared to OS cells on other polymer substrates, however not statistically significant.



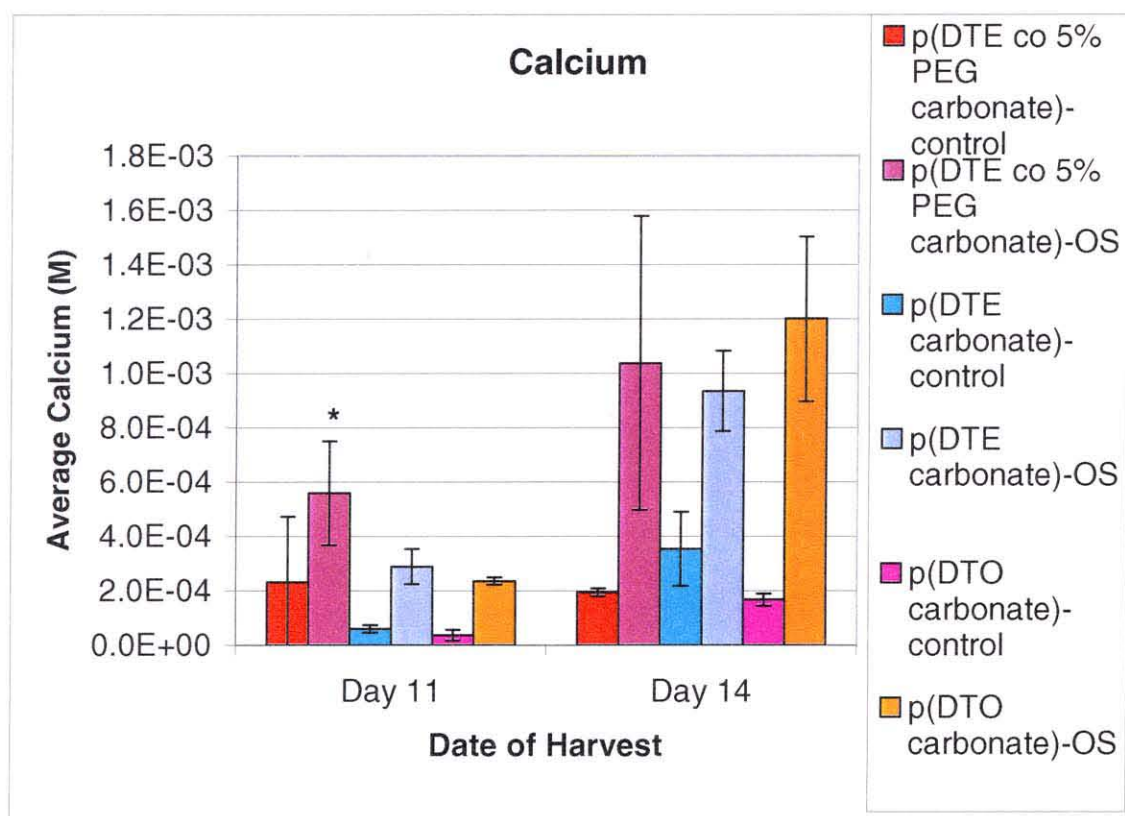
**Figure 4.11** Alkaline Phosphatase activity of undifferentiated hMSCs (control) and OS cells on polymer substrates.



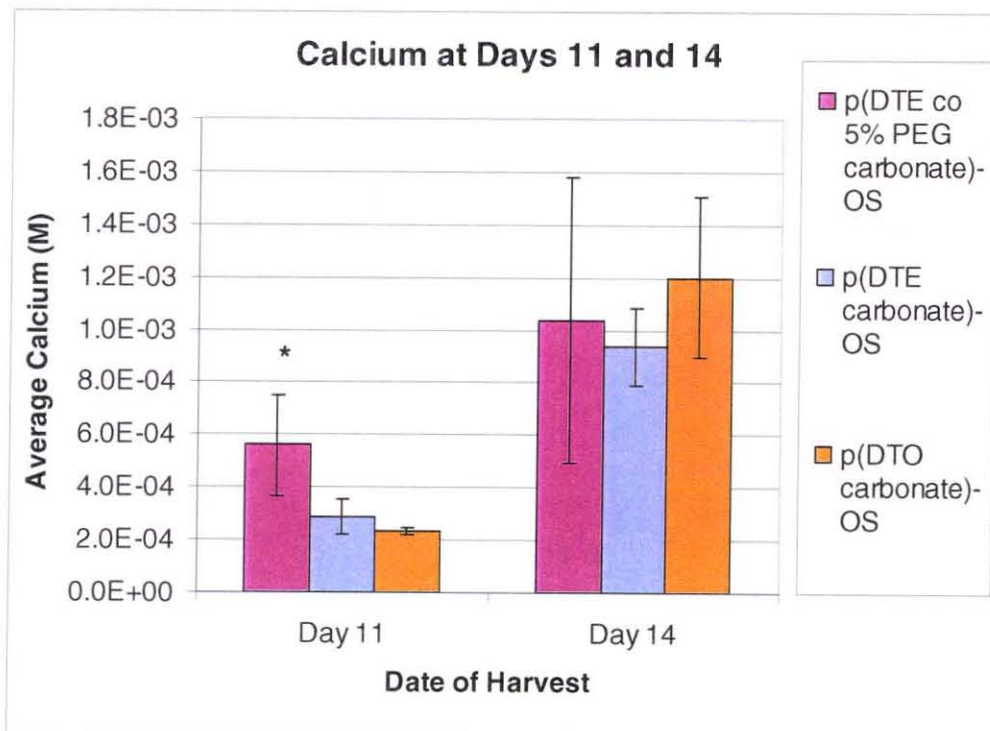
**Figure 4.12** Alkaline Phosphatase activity of OS cells on polymer substrates at days 11 and 14.

### 4.3.3 Calcium

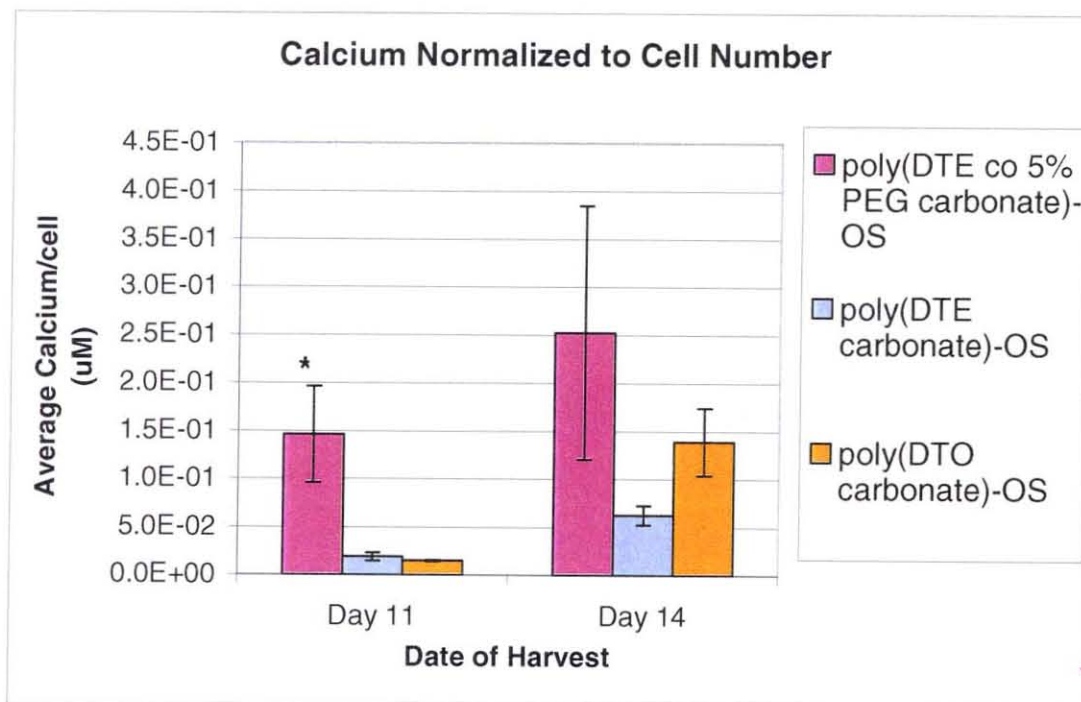
Calcium production is a marker for osteogenic differentiation. Therefore, in this experiment, undifferentiated hMSCs produced less calcium compared to OS cells (Figure 4.13). At day 11, calcium production of OS cells on poly(DTE co 5% PEG-1K carbonate) was statistically higher than poly(DTE carbonate) and poly(DTO carbonate) (Figure 4.14). Calcium production was normalized to the average cell number obtained from the cell proliferation assay (Figure 4.15). At day 11, calcium production normalized to cell number of OS cells on poly(DTE co 5% PEG-1K carbonate) was statistically higher than poly(DTE carbonate) and poly(DTO carbonate).



**Figure 4.13** Calcium production of undifferentiated hMSCs (control) and OS cells on polymer substrates at days 11 and 14. Asterisks indicates a statistically significant difference ( $p < 0.05$ ), between OS cells on poly(DTE co 5% PEG-1K carbonate) to OS cells on both poly(DTE carbonate) and poly(DTO carbonate).



**Figure 4.14** Calcium production of OS cells on polymer substrates. Asterisks indicates a statistically significant difference ( $p < 0.05$ ) between poly(DTE co 5% PEG-1K carbonate) to both poly(DTE carbonate) and poly(DTO carbonate).



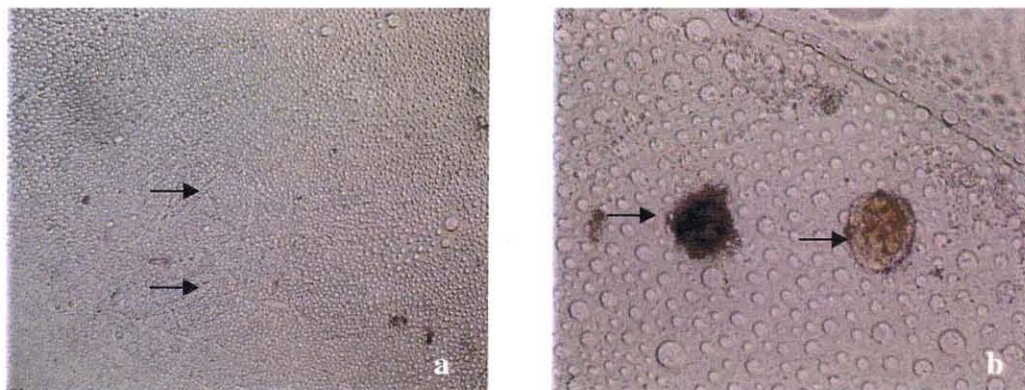
**Figure 4.15** Calcium production normalized to cell number of OS cells on polymer substrates. Asterisks indicate statistical difference ( $p < 0.05$ ), between poly(DTE co 5% PEG-1K carbonate) to both poly(DTE carbonate) and poly(DTO carbonate).

#### **4.4 Effect of PEG on Osteogenic Differentiation**

The data presented thus far demonstrate the anti-cell attachment effect on poly(DTE co 5% PEG-1K carbonate) as well as its effect on osteogenic differentiation markers. The goal of this section was to determine the effect of the concentration of PEG on osteogenic differentiation using poly(DTE co 3% PEG-1K carbonate) and poly(DTE co 5% PEG-1K carbonate). Biochemical assay results were compared to poly(DTE carbonate) which was conducted in a separate experiment in July 2005 (see section 4.3). Below are the results of a 96 well plate experiment conducted in April 2006 using hMSC, Donor 2, passage 2. Cells were seeded on poly(DTE co 3% PEG-1K carbonate) and poly(DTE co 5% PEG-1K carbonate) to determine the effect of the concentration of PEG on osteogenic differentiation. Data analysis was conducted using the Tukey-Kramer test between the polymer substrates.

##### **4.4.1 Cell Morphology**

The modification of the molar fraction of PEG in the polycarbonate backbone, from 3% to 5%, has a substantial effect on the cell morphology. The morphology of OS cells on poly(DTE co 3% PEG-1K carbonate) by day 14 is a confluent monolayer, while the morphology of OS cells on poly(DTE co 5% PEG-1K carbonate) by day 14 is exhibited by the formation of cell aggregates.

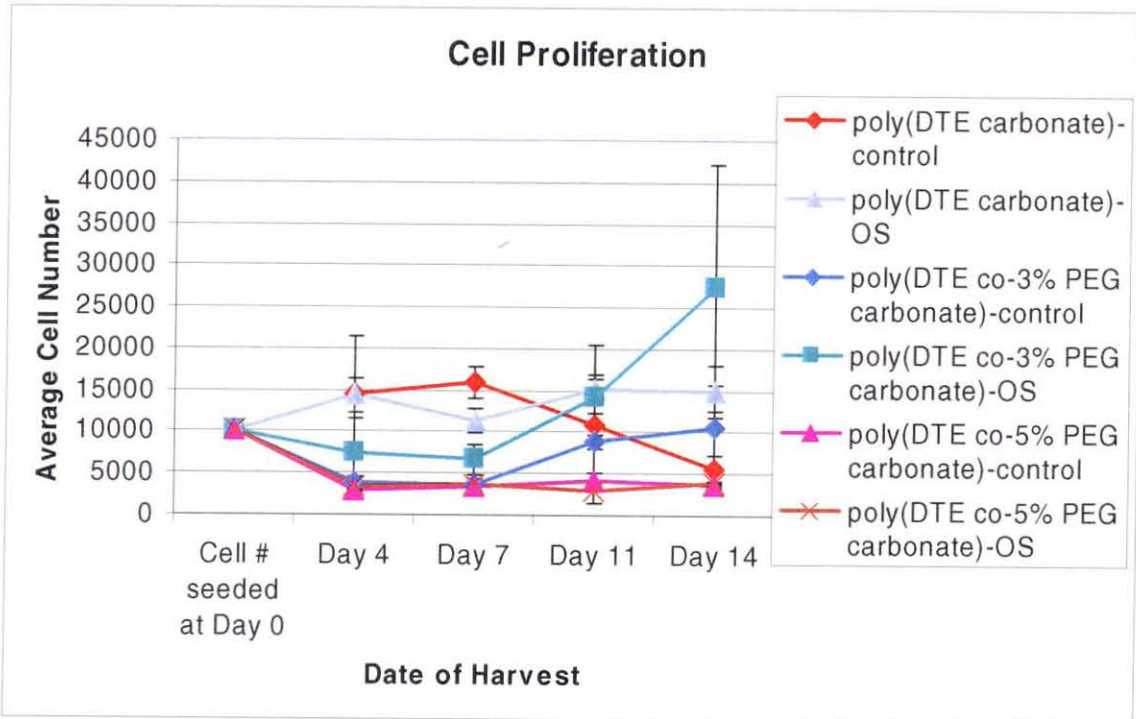


**Figure 4.16** OS cells at day 14: (a) on poly(DTE co 3% PEG-1K carbonate), 10X (b) on poly(DTE co 5% PEG-1K carbonate), 20X. Arrows on (a) indicate cuboidal cell morphology. Arrows on (b) indicate cell aggregation

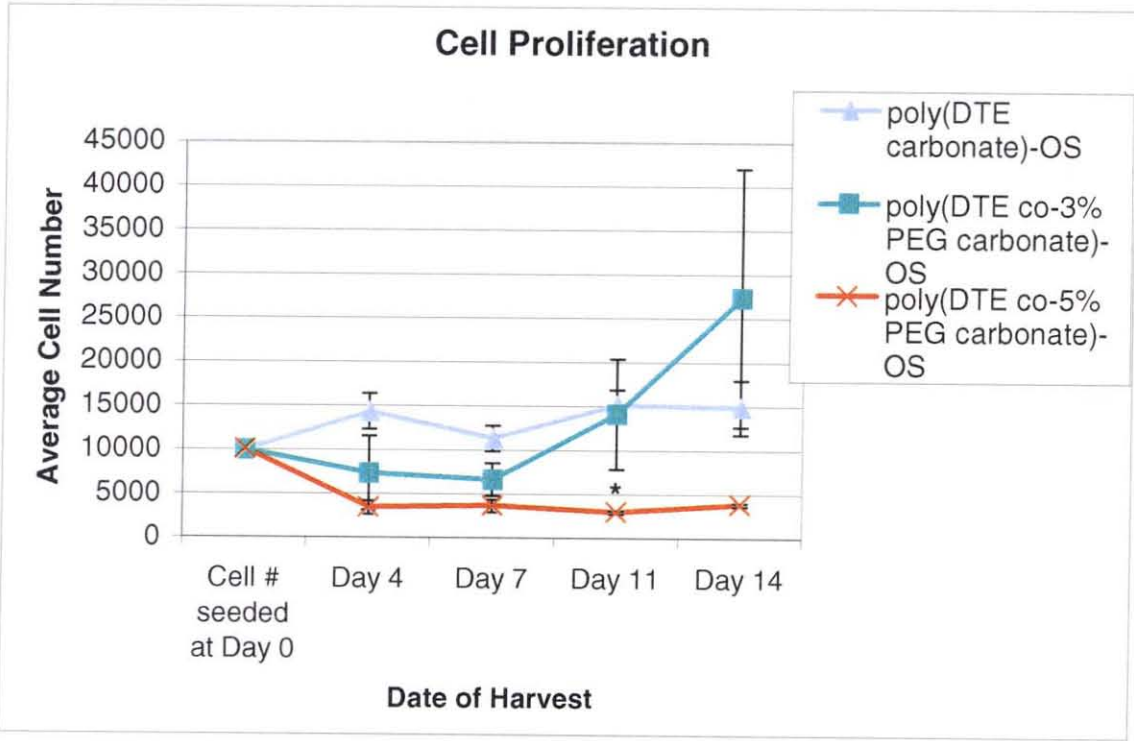
#### 4.4.2 Cell Proliferation

The modification of the molar fraction of PEG in the polycarbonate backbone, from 5% to 3%, has a positive effect on cell proliferation. OS cells on poly(DTE co 3% PEG-1K carbonate) had a higher cell proliferation compared to OS cells on poly(DTE co 5% PEG-1K carbonate) (Figure 4.17). At day 11, OS cell number on poly(DTE co 3% PEG-1K carbonate) is statistically higher than cell number on poly(DTE co 5% PEG-1K carbonate) (Figure 4.18).





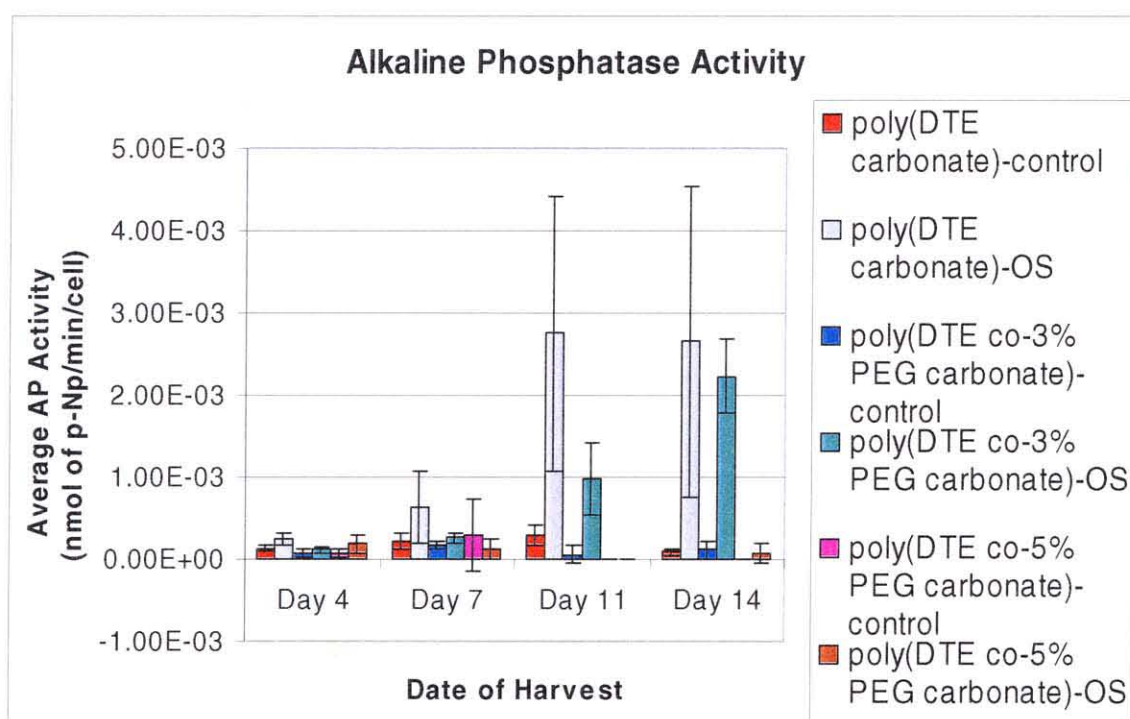
**Figure 4.17** Cell proliferation of undifferentiated hMSCs (control) and OS cells on polymer substrates.



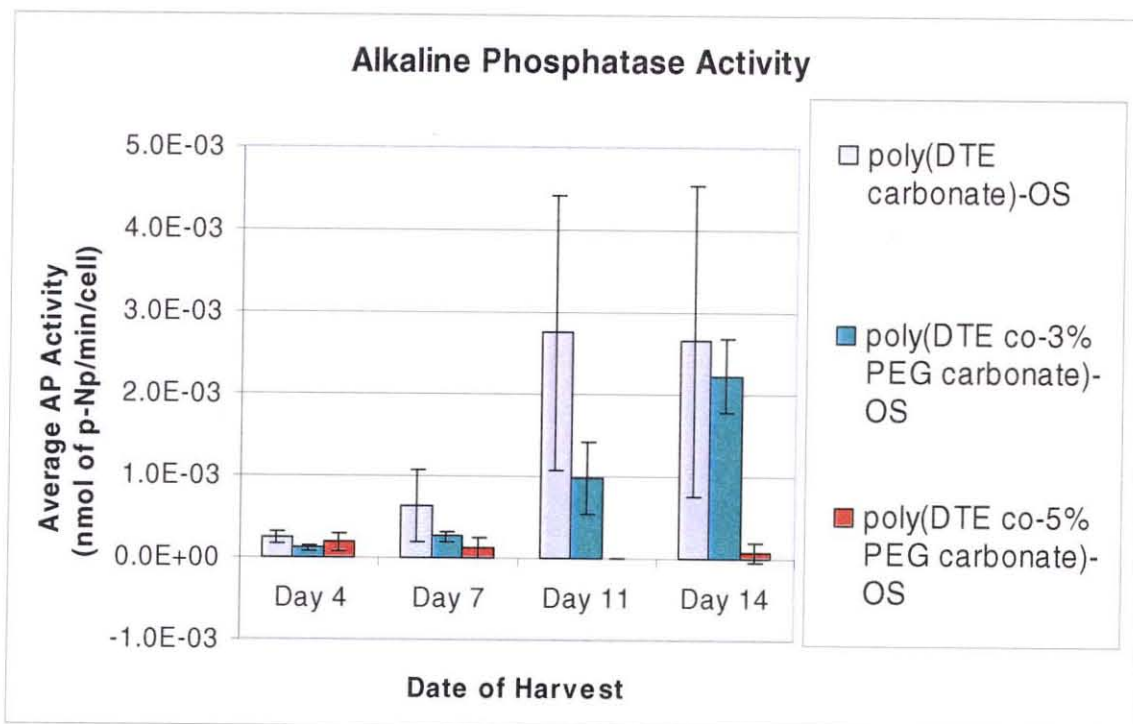
**Figure 4.18** Cell proliferation of OS cells on polymer substrates. Asterisks indicate statistical difference ( $p < 0.05$ ), between poly(DTE co 5% PEG-1K carbonate) to both poly(DTE carbonate) and poly(DTE co 3% PEG-1K carbonate).

### 4.4.3 Alkaline Phosphatase

The modification of the molar fraction of PEG in the polycarbonate backbone, from 5% to 3%, has a positive effect in alkaline phosphatase activity at days 11 and 14. Undifferentiated hMSCs had lower alkaline phosphatase activity compared to OS cells (Figure 4.19). By day 11, there is negligible alkaline phosphatase activity in OS cells on poly(DTE co 5% PEG-1K carbonate). OS cells on (DTE co 3% PEG-1K carbonate) have a higher alkaline phosphatase activity compared to cells on poly(DTE co 5% PEG-1K carbonate) (Figure 4.20).



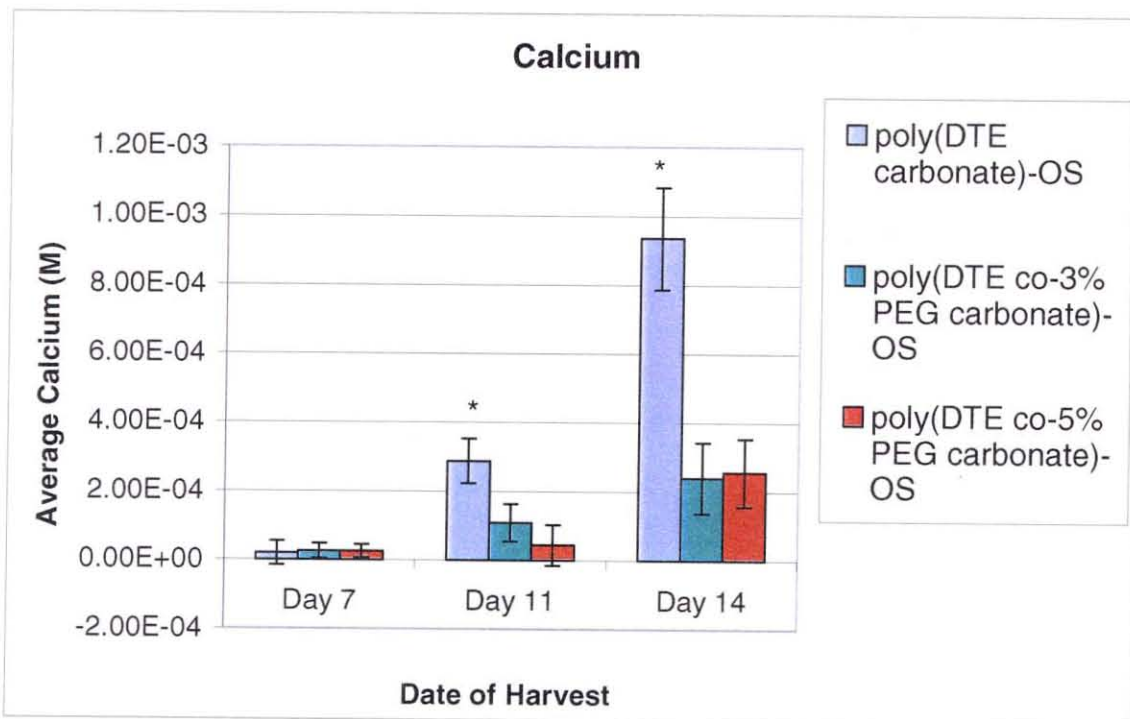
**Figure 4.19** Alkaline Phosphatase activity of undifferentiated hMSCs (control) and OS cells on polymer substrates.



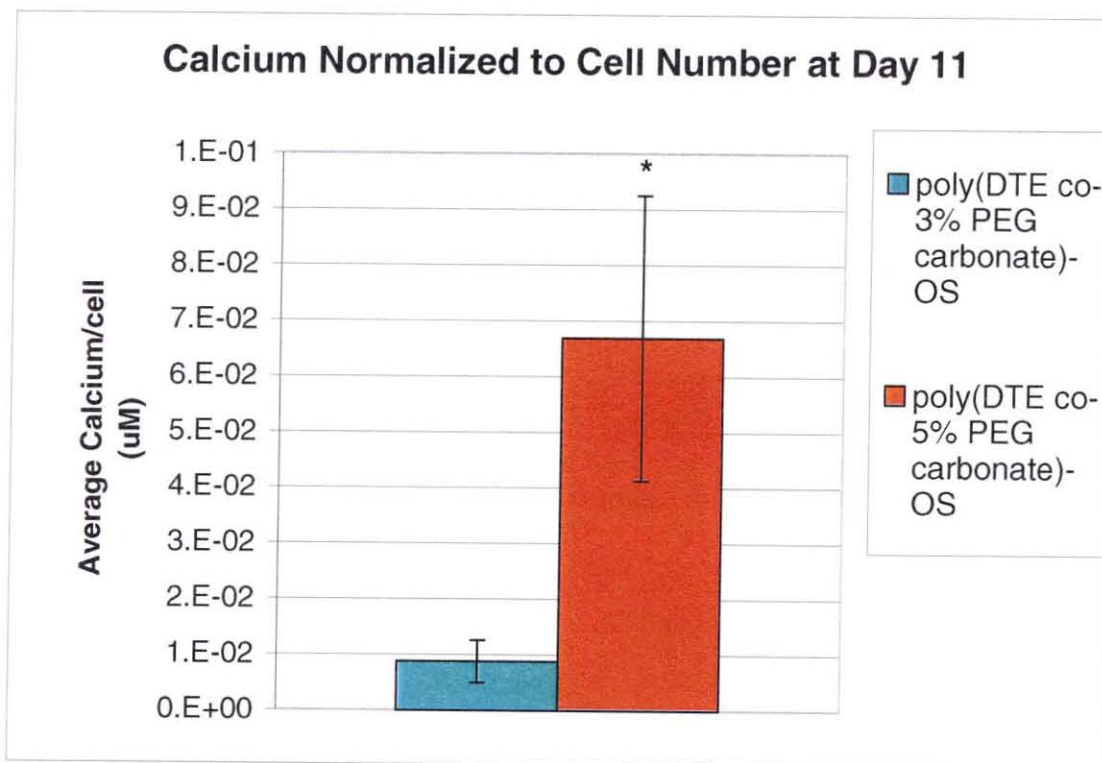
**Figure 4.20** Alkaline phosphatase activity of OS cells on polymer substrates.

#### 4.4.4 Calcium

The modification of the molar fraction of PEG in the polycarbonate backbone, from 3% to 5%, has an increased effect on calcium production at day 14 (Figure 4.21). OS cells on poly(DTE co 5% PEG-1K carbonate) had a statistically higher production of calcium per cell compared to OS cells on poly(DTE co 3% PEG-1K carbonate) (Figure 4.22).



**Figure 4.21** Calcium production of OS cells on polymer substrates. Asterisks indicate a statistical difference ( $p < 0.05$ ), between OS cells on poly(DTE carbonate) compared to poly(DTE co 3% PEG-1K carbonate) and poly(DTE co 5% PEG-1K carbonate).



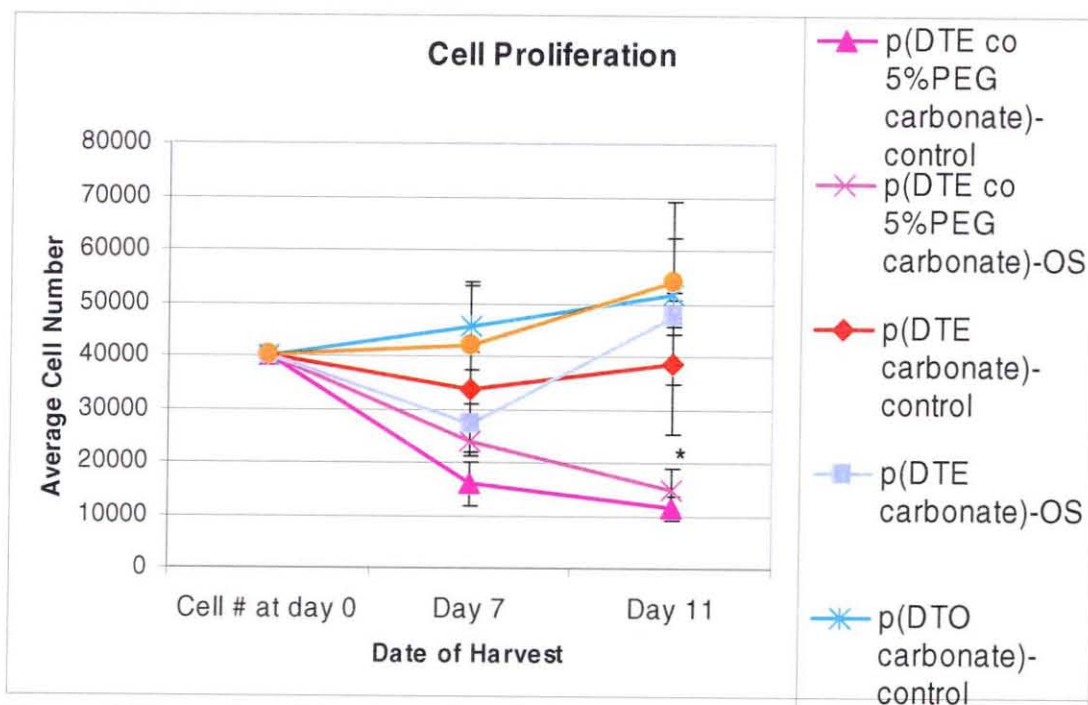
**Figure 4.22** Calcium normalized to cell number of OS cells on poly(DTE co 3% PEG-1K carbonate). Asterisks indicated statistical significance ( $p < 0.05$ ).

## **4.5 Biochemical Assays: 24 Well Plate Experiment**

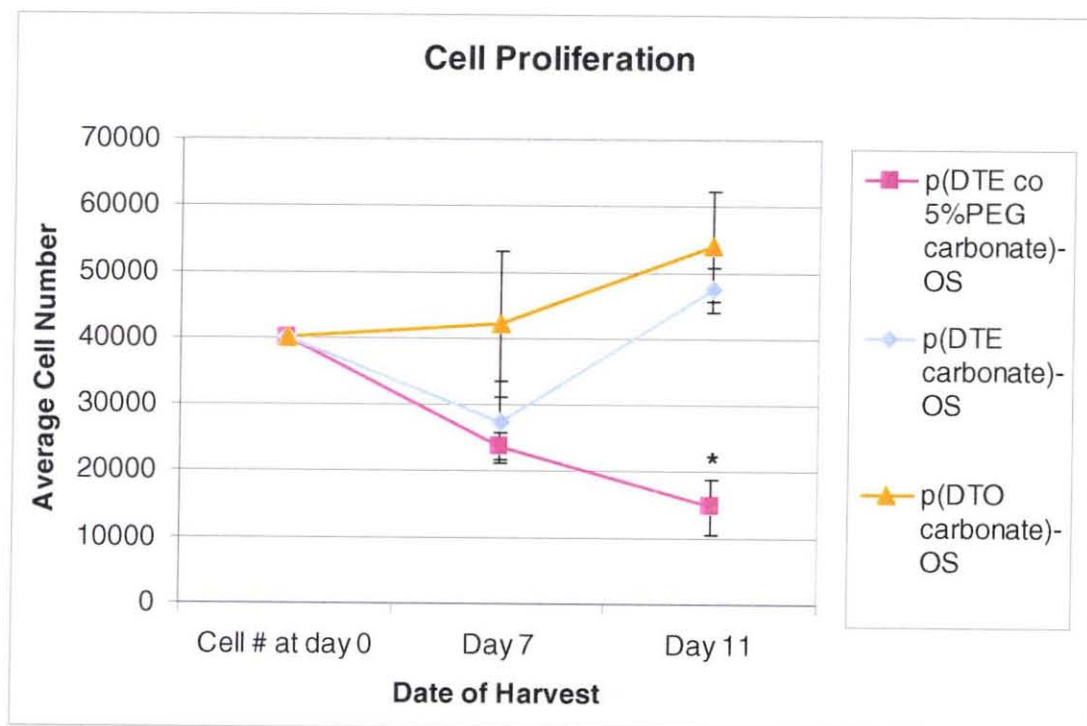
The following section is the quantitative representation of osteogenic differentiation in terms of cell proliferation, alkaline phosphatase activity and calcium production on spin coated disks in 24 well plates. Below are the results of an experiment conducted in December 2005 using hMSC, Donor 2, passage 4. For each experiment, two graphs are represented: one representing undifferentiated hMSCs and osteogenic differentiated hMSCs (OS cells) (Figures 4.23, 4.25, 4.27) and the other representing only OS cells (Figures 4.24, 4.26, 4.28, 4.29). Data analysis was conducted using the Tukey-Kramer test between the polymer substrates.

### **4.5.1 Cell Proliferation**

Cells were harvested at days 7 and 11 for DNA assay. At day 11, proliferation of OS cells on poly(DTE co 5% PEG-1K carbonate) is statistically lower than both poly(DTE carbonate) and poly(DTO carbonate). (Figure 4.23 and Figure 4.24)



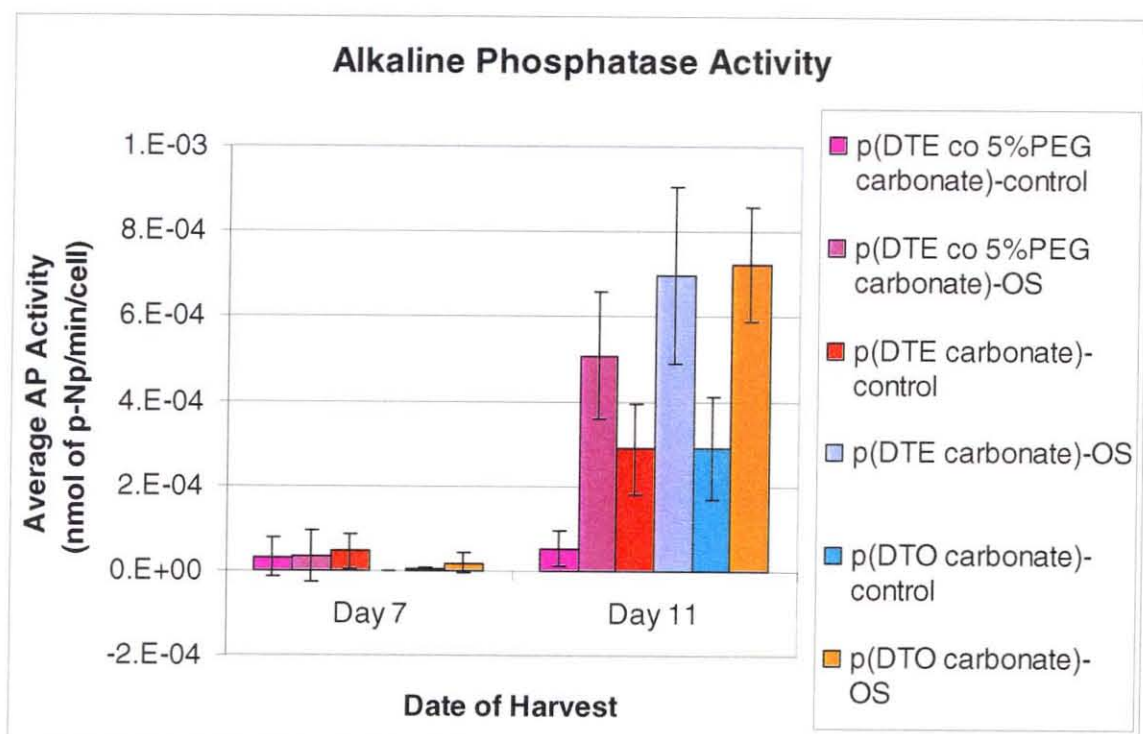
**Figure 4.23** Cell proliferation of undifferentiated hMSCs (control) and OS cells on polymer substrates in a 14-day culture. Asterisks indicates a statistically significant difference ( $p < 0.05$ ), between OS cells on poly(DTE co 5% PEG-1K carbonate) to OS cells on both poly(DTE carbonate) and poly(DTO carbonate).



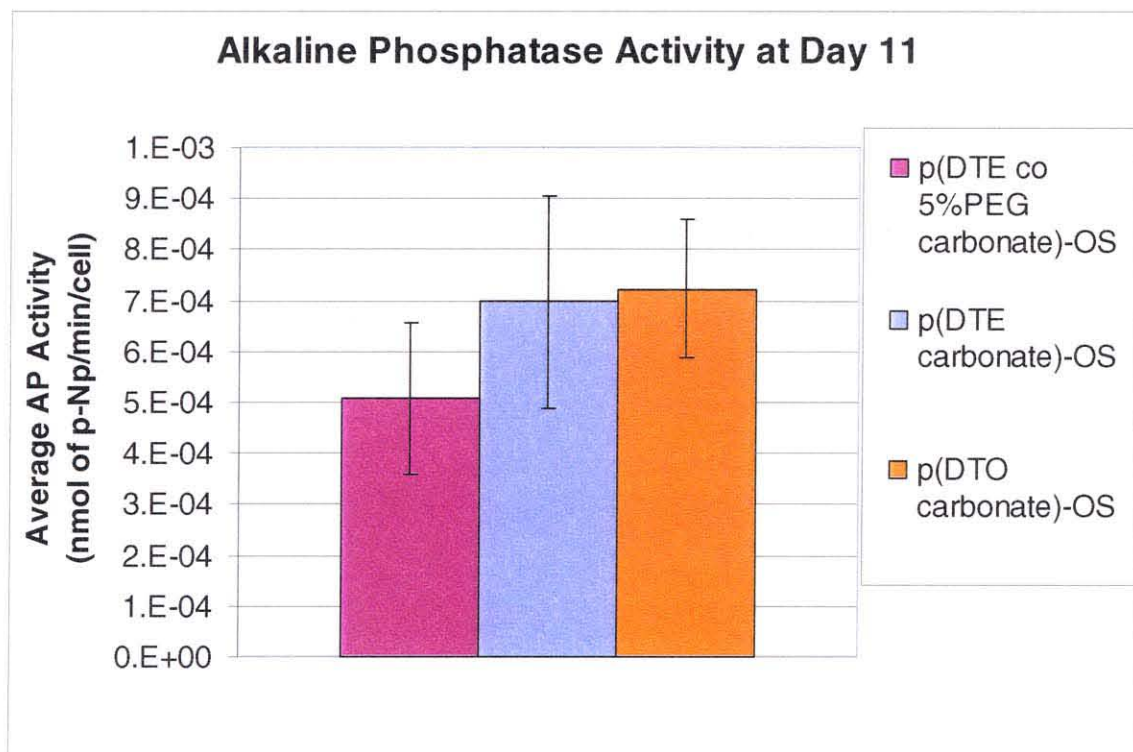
**Figure 4.24** Cell proliferation of OS cells on polymer substrates. Asterisks indicates a statistically significant difference ( $p < 0.05$ ) between poly(DTE co 5% PEG-1K carbonate) to both poly(DTE carbonate) and poly(DTO carbonate).

### 4.5.2 Alkaline Phosphatase

At day 7, alkaline phosphatase activity was negligible. Undifferentiated hMSCs had lower alkaline phosphatase activity compared to OS cells (Figure 4.25). Alkaline phosphatase activity of OS cells was not statistically different between substrates at day 11 (Figure 4.26).



**Figure 4.25** Alkaline Phosphatase activity of undifferentiated hMSCs (control) and OS cells on polymer substrates.

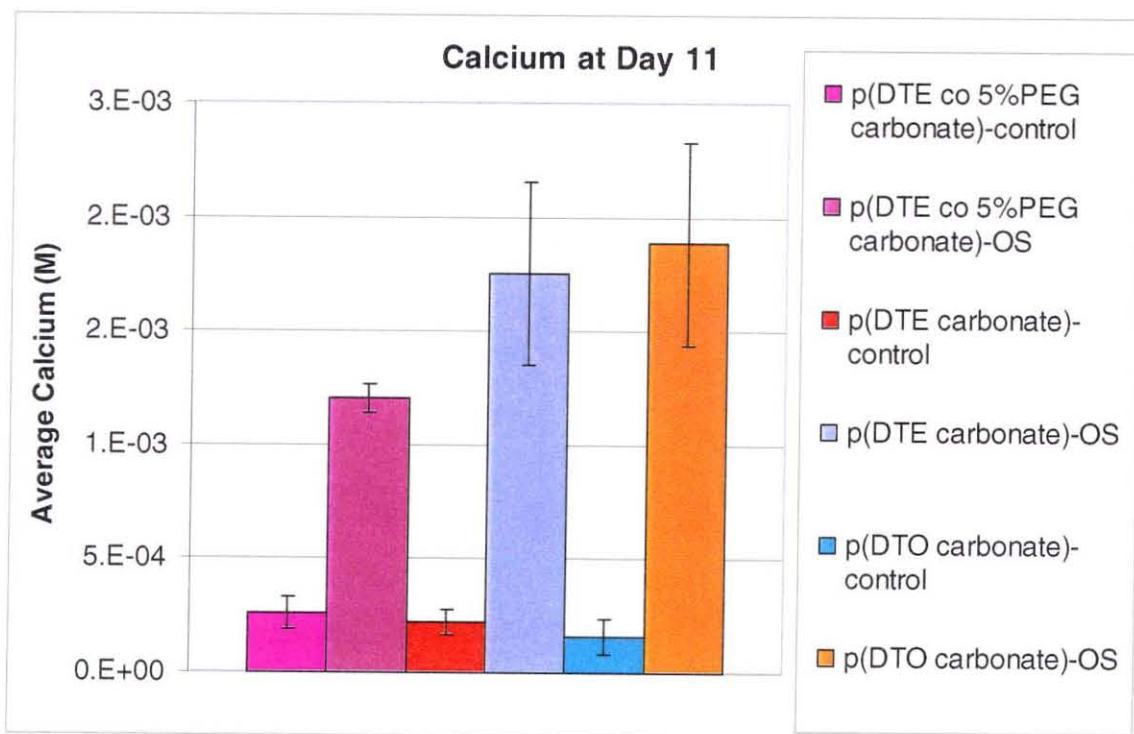


**Figure 4.26** Alkaline Phosphatase activity of OS cells on polymer substrates.

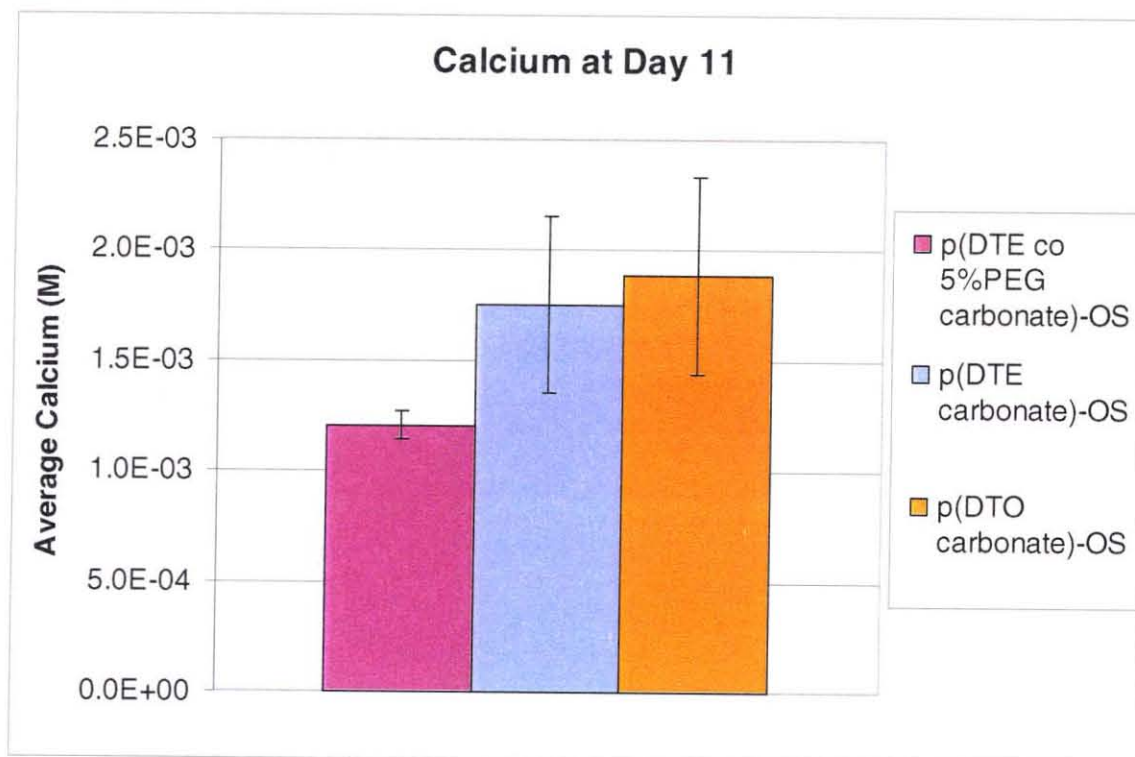
#### 4.5.3 Calcium

Undifferentiated hMSCs produced much less calcium than osteogenic differentiated hMSCs (Figure 4.27). There was no statistical difference in calcium production of OS cells between the polymer substrates (Figure 4.28). Calcium production was normalized to the average cell number obtained from the cell proliferation assay. At day 11, calcium production normalized to cell number of OS cells on poly(DTE co 5% PEG-1K carbonate) was statistically higher than poly(DTE carbonate) and poly(DTO carbonate) (Figure 4.29). This effect was also observed in the 96 well experiment (Figure 4.15).

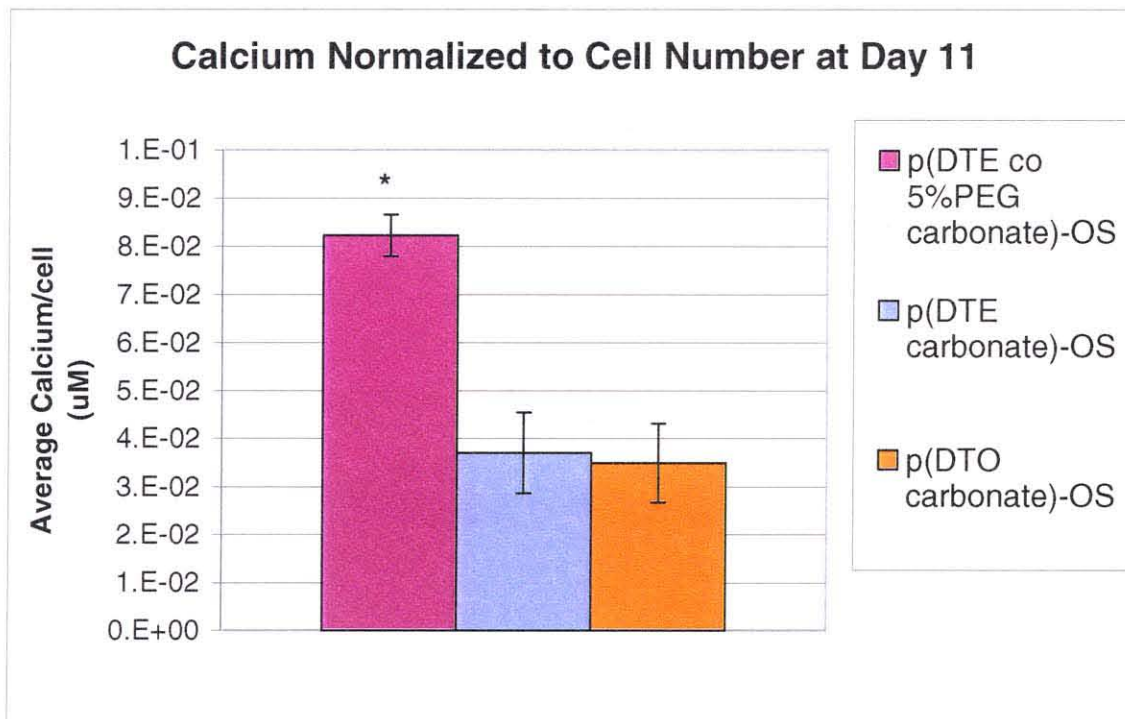




**Figure 4.27** Calcium production of undifferentiated hMSCs (control) and OS on polymer substrates.



**Figure 4.28** Calcium production of OS cells on polymer substrates.

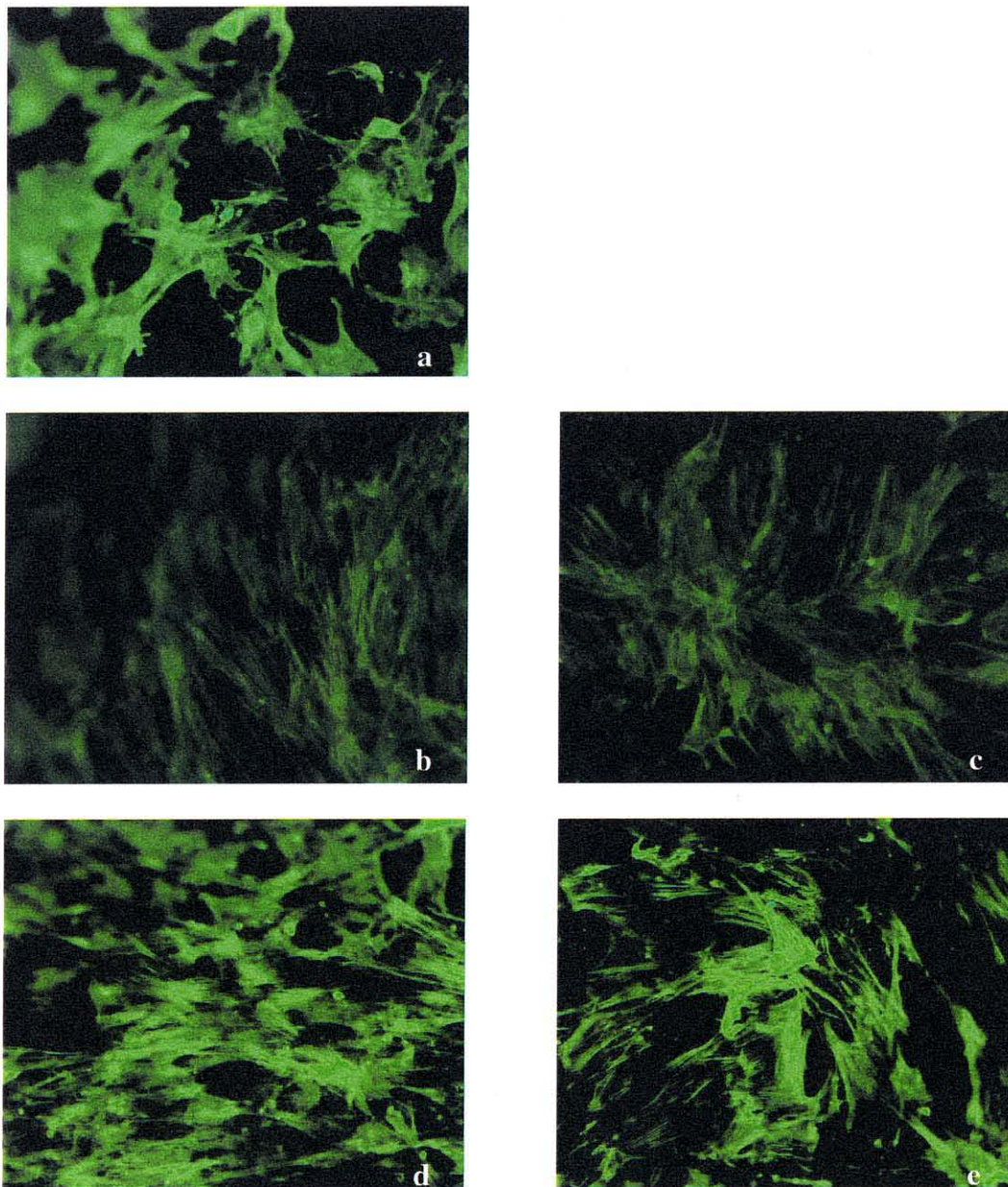


**Figure 4.29** Calcium production normalized to cell number of OS cells. Asterisks indicates a statistically significant difference ( $p < 0.05$ ), between poly(DTE co 5% PEG-1K carbonate) to both poly(DTE carbonate) and poly(DTO carbonate).

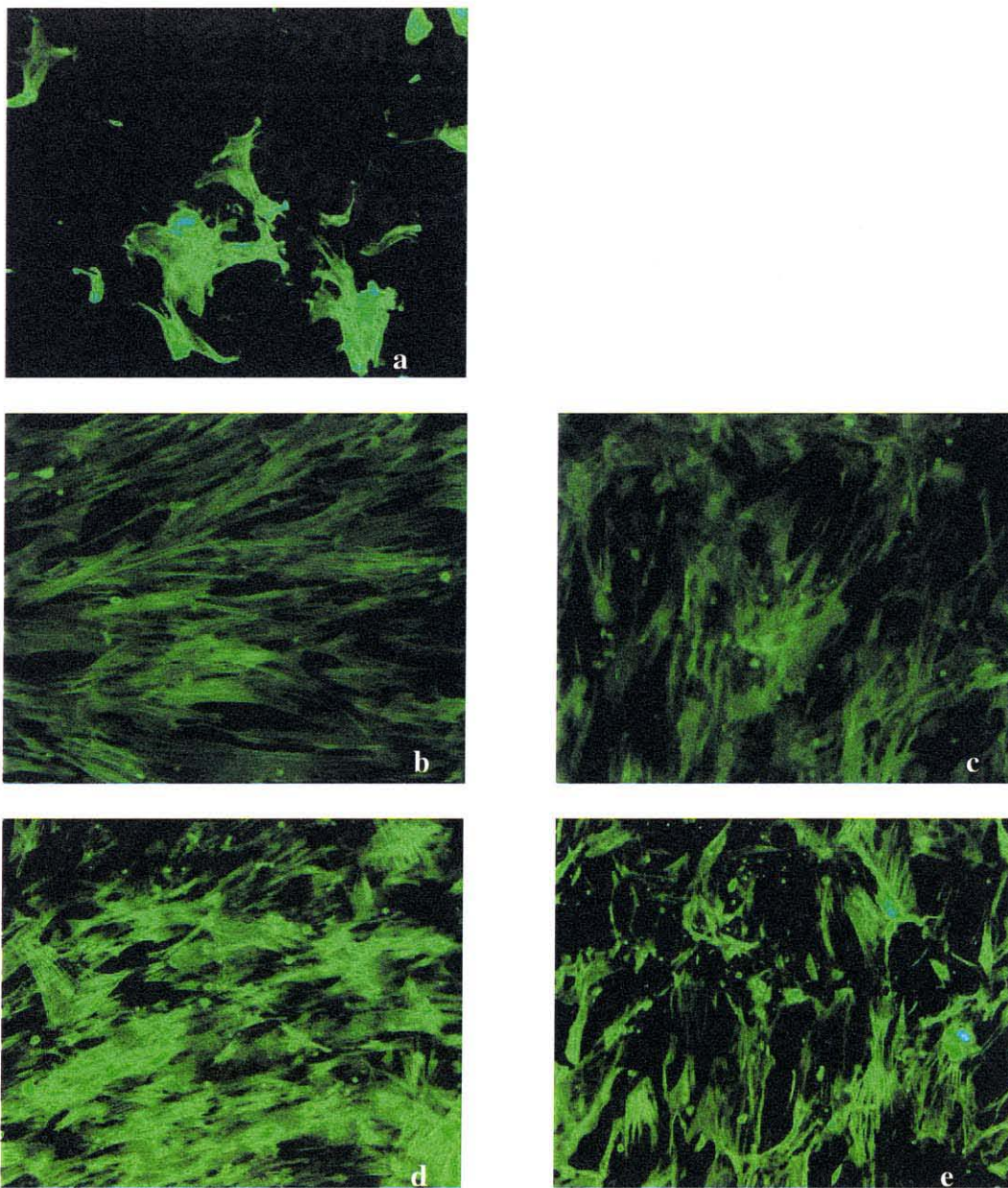
#### 4.6 Cytoskeleton Staining

Cytoskeleton staining is an indication of focal adhesion contacts onto the substrate. In this experiment, there were differences in the focal adhesion contacts of undifferentiated hMSCs and osteogenic differentiated hMSCs (OS cells) on the various substrates. The actin cytoskeleton assembly of undifferentiated hMSCs has a parallel orientation (Figures 4.30d, 4.31d, 4.33d). However, the actin cytoskeleton assembly of OS cells has a mesh-like orientation (Figures 4.30e, 4.31e, 4.33e). As early as 5 hours, cells have established adhesion contacts with the polymer substrates (Figure 4.30a, 4.31a, and 4.33a). Cells on poly(DTE co 5% PEG-1K carbonate) did not establish focal adhesion contacts to the substrate (Figure 4.32). There was no actin filaments observed in cells seeded on

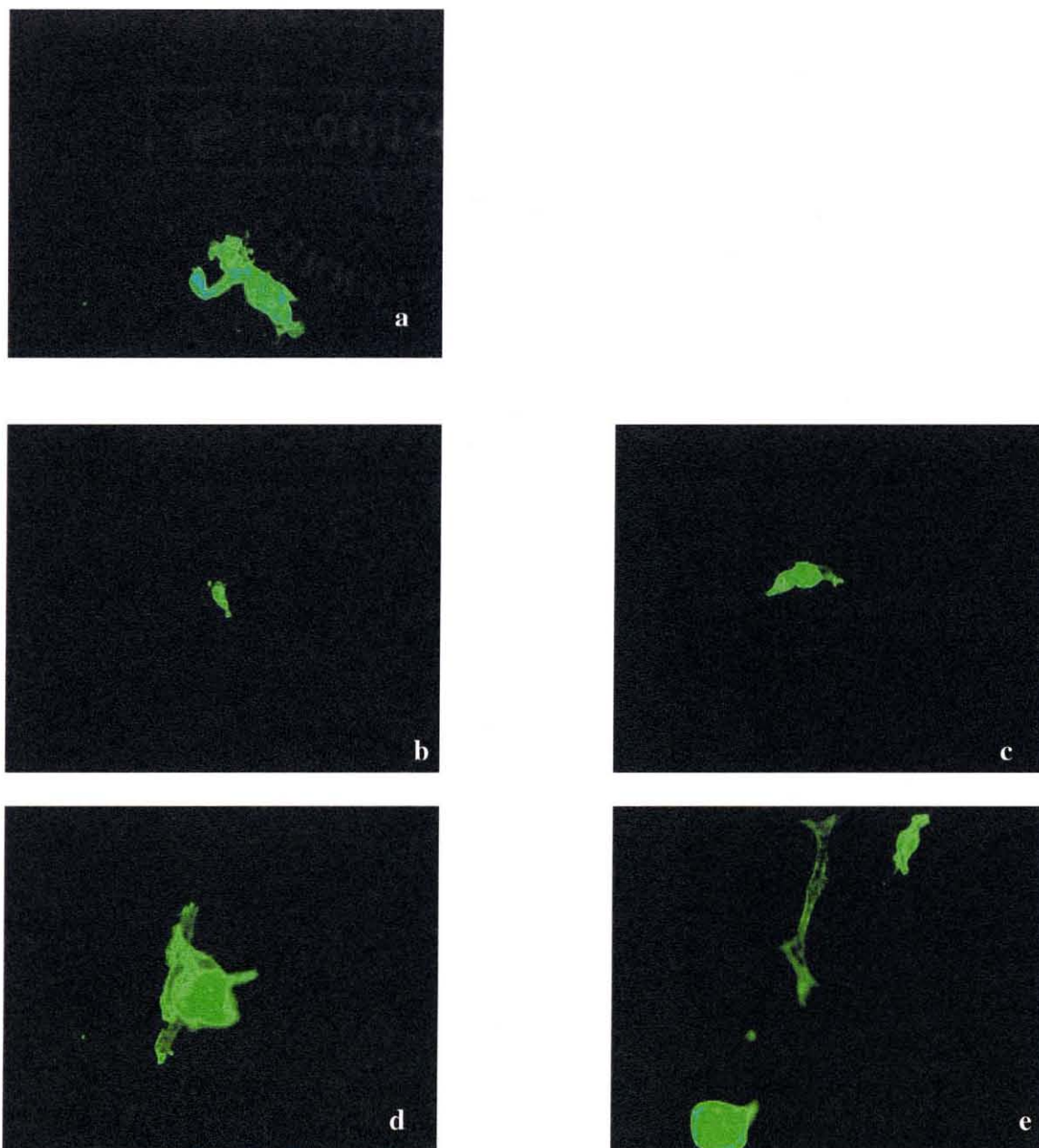
poly(DTE co 5% PEG carbonate). Images shown here were taken at 5 hours, 4 days and 7 days after initial seeding at a magnification of 20X.



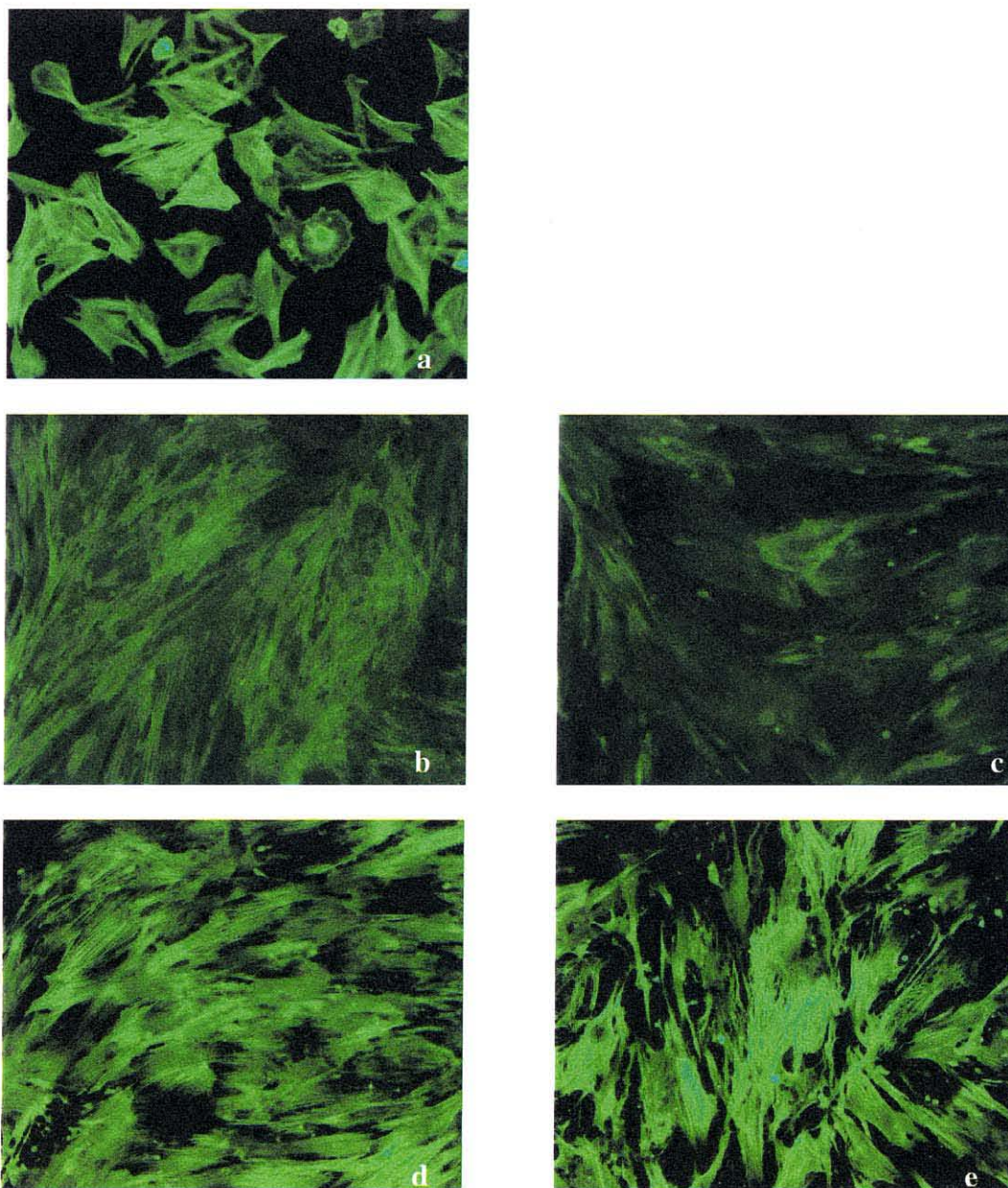
**Figure 4.30** Cells on poly(DTE carbonate), 20X: (a) undifferentiated hMSCs on poly(DTE carbonate) 5 hours after seeding, (b) undifferentiated hMSCs on poly(DTE carbonate) at day 4, (c) OS cells on poly(DTE carbonate) at day 4, (d) undifferentiated hMSCs on poly(DTE carbonate) at day 7, (e) OS cells on poly(DTE carbonate) at day 7.



**Figure 4.31** Cells on poly(DTO carbonate), 20X: (a) undifferentiated hMSCs on poly(DTO carbonate) 5 hours after seeding, (b) undifferentiated hMSCs on poly(DTO carbonate) at day 4, (c) OS cells on poly(DTO carbonate) at day 4, (d) undifferentiated hMSCs on poly(DTE carbonate) at day 7, (e) OS cells on poly(DTE carbonate) at day 7.



**Figure 4.32** Cells on poly(DTE co 5% PEG-1K carbonate), 20X: (a) undifferentiated hMSCs on poly(DTE co 5% PEG-1K carbonate) at 5 hours after seeding, (b) undifferentiated hMSCs on poly(DTE co 5% PEG-1K carbonate) at day 4, (c) OS cells on poly(DTE co 5% PEG-1K carbonate) at day 4, (d) undifferentiated hMSCs on poly(DTE co 5% PEG-1K carbonate) at day 7, (e) OS cells on poly(DTE co 5% PEG-1K carbonate) at day 7.

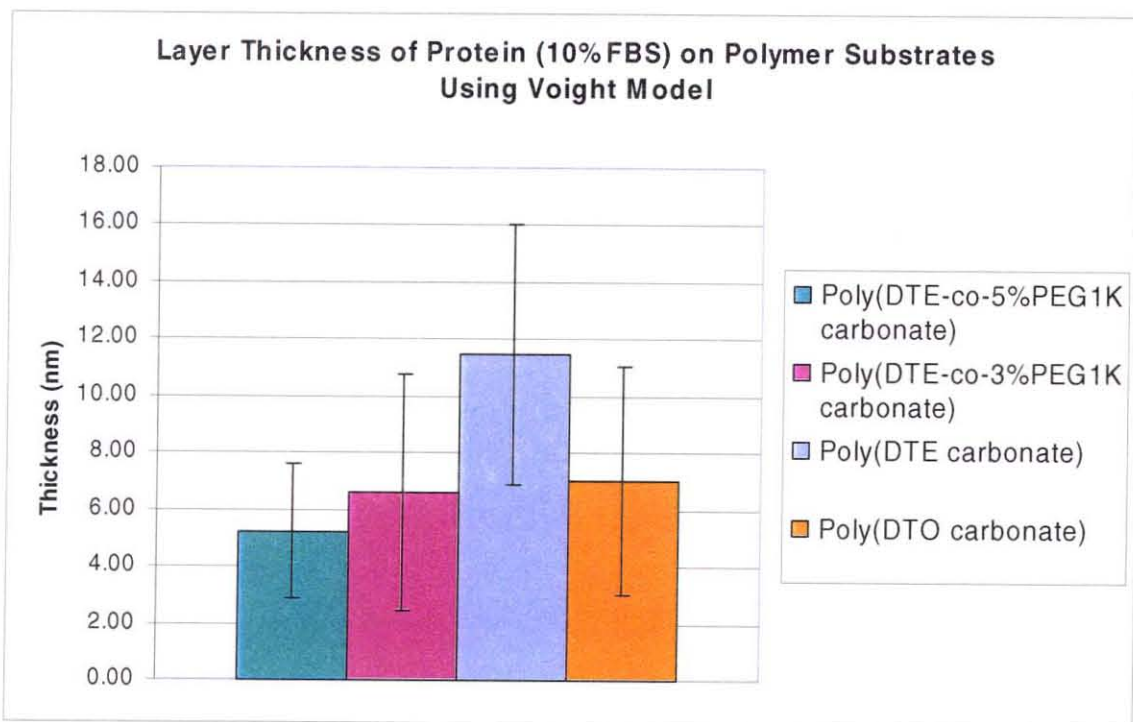


**Figure 4.33** Cells on tissue culture polystyrene (TCPS), 20X: (a) undifferentiated hMSCs on TCPS 5 hours after seeding, (b) undifferentiated hMSCs on TCPS at day 4, (c) OS cells on TCPS at day 4, (d) undifferentiated hMSCs on TCPS at day 7, (e) OS cells on TCPS at day 7.

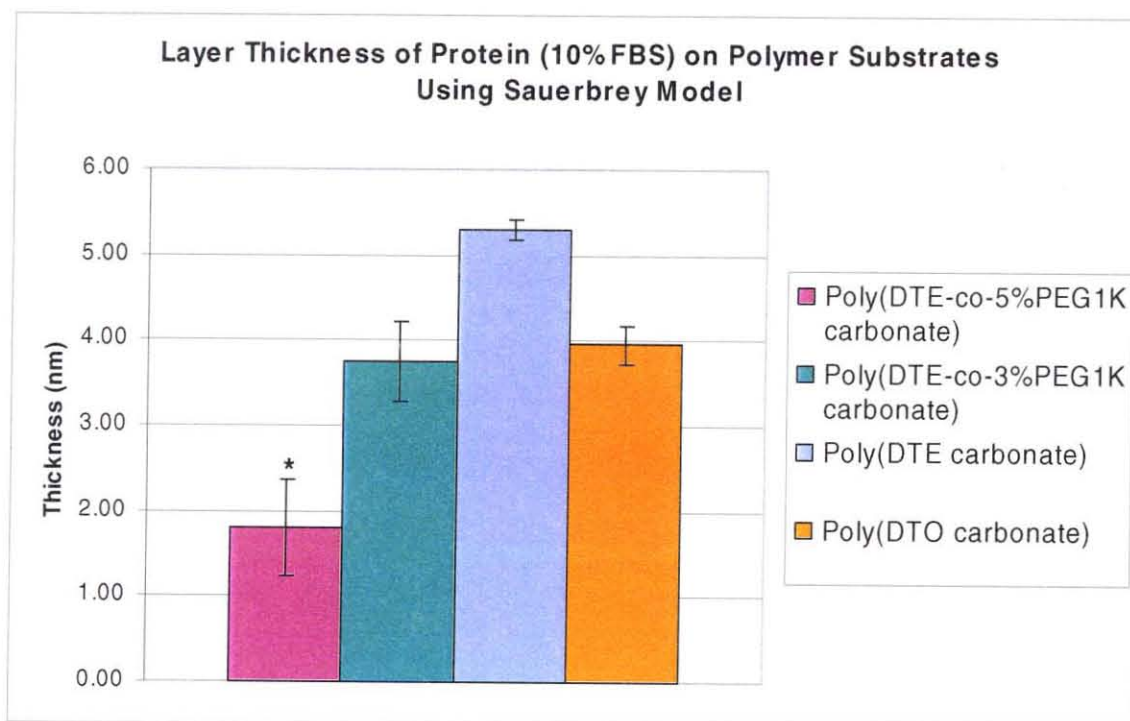
## 4.7 QCM-D

The QCM-D data provides information about the thickness of the adsorbed protein layer. The data for the change in frequency and dissipation was modeled to using 2 different models: Voight and Sauerbrey. The Sauerbrey model is used to calculate the adsorbed mass of thin, non-dissipative layers of protein, while the Voight model is used to calculate the adsorbed mass of a thick dissipative layer of protein. In this particular application, the Voight model is preferred.

With 10% FBS, there is no statistical difference in the thickness of the protein layer between the substrates using the Voight model due to the high standard deviations (Figure 4.34). However, the thickness of the adsorbed protein layer using the Sauerbrey model, shows a statistical difference between poly(DTE co 5% PEG-1K carbonate) and the other substrates (Figure 4.35). Although, the Sauerbrey model is not the preferred model for this application, it can be generally concluded that the adsorbed layer of fetal bovine serum on poly(DTE co 5% PEG-1K carbonate) is thinner compared to the other substrates.



**Figure 4.34** Thickness of adsorbed layer of 10% FBS onto substrates using Voight model.

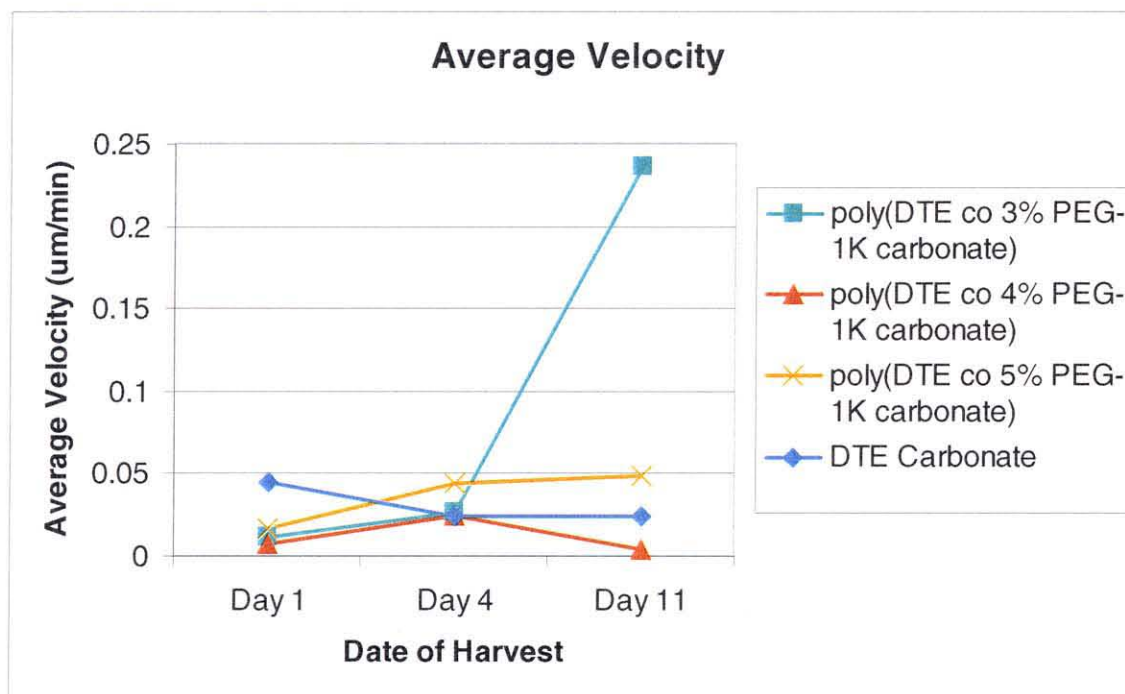


**Figure 4.35** Thickness of adsorbed layer of 10% FBS onto substrates using Sauerbrey model. Asterisks indicate statistical difference ( $p < 0.05$ ) between poly(DTE co 5% PEG-1K carbonate) and other substrates.

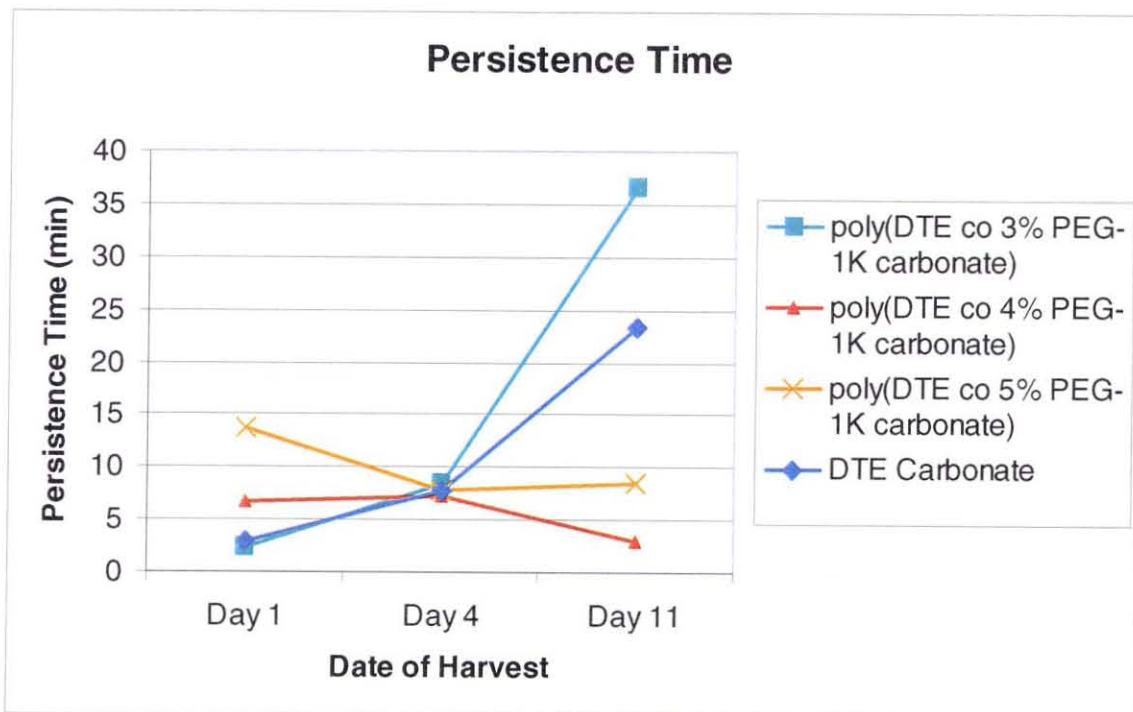


### 4.8 Cell Motility

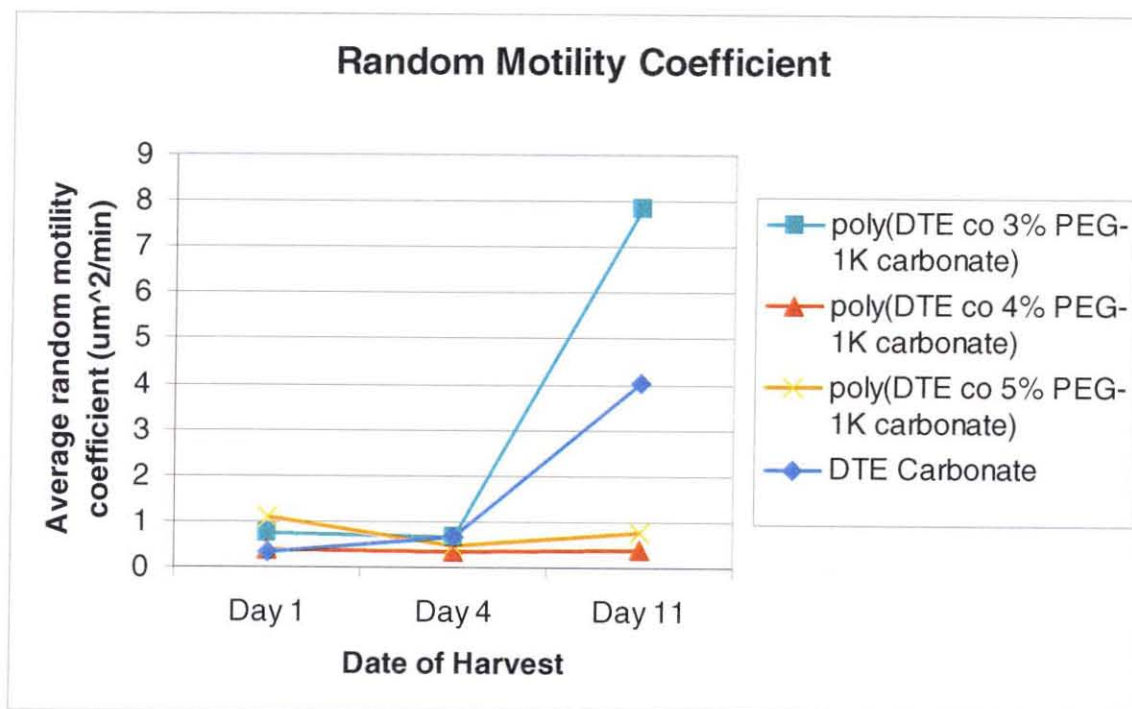
The goal of the cell motility experiment was to evaluate the relationship between cell substrate adhesion and cell-cell aggregation to motility by modifying the molar fraction of PEG in the polycarbonate backbone. The cell motility experiments were conducted in March 2006, using hMSCs, Donor 4, passage 2. The effect of the molar fraction of PEG in the polycarbonate backbone, on motility is evident in this experiment. As osteogenic differentiation proceeds to day 11, OS cells on poly(DTE co 3% PEG-1K carbonate) has an increased effect on random motility coefficient, velocity and persistence time compared to the other substrates (Figures 4.36-4.39). It is interesting to note that the motility data collected at day 11 on poly(DTE co 3% PEG-1K carbonate) is the highest in the experiment. By day 11, there is considerable mineralization of the extracellular matrix.



**Figure 4.36** Average velocity of cells on substrates during 11 day motility experiment.



**Figure 4.37** Persistence time of cells on substrates during 11 day motility experiment.



**Figure 4.38** Average random motility coefficient of cells on substrates during 11 day motility experiment.

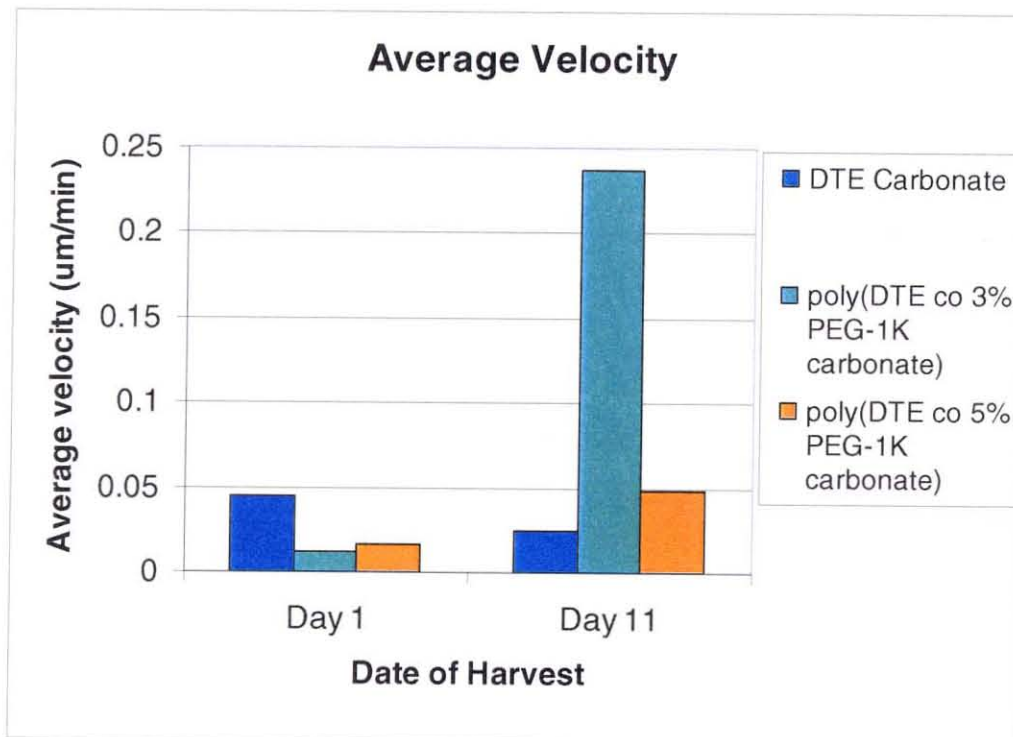


Figure 4.39 Average velocity at day 1 and 11 of cells.

## CHAPTER 5

### DISCUSSION AND FUTURE RESEARCH

The regenerative potential of mesenchymal stem cells (MSCs), along with the relative ease in culturing these cells *in vitro* has made them an attractive candidate in many tissue engineering applications such as bone graft substitutes. Properties of ideal substrates for potential bone graft scaffolds are:

- Bone in-growth
- Porosity
- Mechanical integrity
- Bioresorbable

The bioresorbability and mechanical properties of tyrosine derived polycarbonates have been well characterized, and are appropriate for orthopedic applications. The goal of this study was to determine the extent of osteogenic differentiation of hMSCs on tyrosine derived polycarbonates as a function of the alkyl ester pendent chain length and molar percent of PEG in the polycarbonate backbone.

Tyrosine derived polycarbonates are a library of bioresorbable polymers that possess the same backbone yet differ in the methyl groups of the alkyl ester pendent chain. The polycarbonates that were chosen for this study: poly(DTE carbonate) and poly(DTO carbonate) have two methyl groups and eight methyl groups respectively. Tyrosine derived polycarbonate PEG copolymers have three modifiable parameters: the alkyl ester pendent chain length, the molecular weight of PEG and the molar percent of

PEG. In this study, two parameters remained fixed: the alkyl ester pendent chain (ethyl) and the molecular weight of the PEG molecule (1000 kDa), while the molar percent of PEG was adjusted between 3-5%. The modifications in the polymer chemistry of polycarbonates, whether it is the addition of methyl groups in the alkyl ester pendent chain or the incorporation of PEG in the backbone has a significant effect on the surface wettability of the polymer, which is reflected in the contact angle measurements (Table 4.1). It was hypothesized that the surface wettability may have a negative effect on osteogenic differentiation.

In this study, osteogenic differentiation was assessed with the quantitation of osteoblast biomarkers: alkaline phosphatase, and calcium. Cell proliferation was the determinant of cell attachment and surface biocompatibility. Osteogenic differentiation was also determined by morphology and cytoskeletal organization.

In the preceding results of the biochemical assays on 96 well plates, it was shown that there was no statistical difference in the osteogenic differentiation in terms of cell proliferation, alkaline phosphatase activity, calcium and osteocalcin, between poly(DTE carbonate) and poly(DTO carbonate). However, the presence of PEG in the backbone of the polycarbonate had a profound impact on osteogenic differentiation. PEG has an anti-attachment effect on cells, which is reflected in the decrease in the cell number from the initial cell seeding density (Figure 4.10). There was no statistical difference between the substrates in terms of alkaline phosphatase activity. However, the calcium production of osteogenic differentiated cells normalized to cell number on poly(DTE co 5% PEG-1K carbonate) is statistically greater compared to both poly(DTE carbonate) and poly(DTO carbonate).

There was no qualitative difference in the morphology of OS cells on poly(DTE carbonate) and poly(DTO carbonate). On these substrates at late timepoints (7 and 11 days), OS cells formed a confluent single-cell monolayer. However, on poly(DTE co 5% PEG-1K carbonate) at late timepoints, OS cells were characterized by sparse clusters of cell aggregates (Figure 4.7e). In fact, aggregation was observed as early as 4 hours after cell seeding (Figure 4.7a).

The cytoskeleton organization through actin-filament staining, indicates the focal adhesion contacts between the cell and the substrates and is representative of the integrin-ligand binding interactions. The ligand in this case, are the adsorbed proteins present in the cell culture medium, onto the substrate. In this study, it was observed that cytoskeletal organization of OS cells on the substrates: poly(DTE carbonate) and poly(DTO carbonate) had a mesh-like orientation (Figure 4.30e and Figure 4.31e), in contrast the cytoskeletal organization of hMSCs on these substrates had a parallel orientation (Figure 4.30d and Figure 4.31d). The cytoskeletal organization of cells grown on poly(DTE co 5% PEG-1K carbonate), (Figure 4.32) was not apparent, therefore concluding that these cells did not form focal adhesion contacts to the substrates. Another interesting point is that the morphology and cytoskeletal organization of cells grown on poly(DTE co 5% PEG-1K carbonate) did not change from the mesenchymal stem cell to the osteogenic differentiated cell. These observations suggest that aggregation, a cell-cell cohesive force is much stronger than the cell-substrate adhesive force on polycarbonates containing 5% PEG.

The biochemical profile, morphology and cytoskeletal organization of OS cells on poly(DTE co 5% PEG-1K carbonate) is remarkably different compared to cells on the

other polymer substrates. This could be due to an accelerated state of differentiation. The differentiation of osteoblasts proceeds with proliferation, which decreases, as matrix maturation and mineralization occurs[16]. The absence of proliferation and increased mineralization of OS cells on poly(DTE co 5% PEG-1K carbonate) could be evidence of a highly differentiated osteoblast. Further biochemical assays testing for the presence of osteopontin must be done to prove this.

The phenomenon of cell aggregation versus cell-substrate adhesion and migration on polycarbonates containing PEG was reported by Ryan et al, in which L929 mouse fibroblasts were transfected with different cadherin clones and seeded on polycarbonates containing various concentrations of PEG. It was shown that decreasing the molar percent of PEG in polycarbonates increases the cell-substrate adhesion as well as increasing the rate of emigration[34]. In this study, this phenomenon was observed on cells seeded on poly(DTE co 3% PEG-1K carbonate) which were morphologically more spread out than cells seeded on poly(DTE co 5% PEG-1K carbonate) (Figure 4.16), and had a higher incidence of cell attachment indicated by the cell proliferation data. The alkaline phosphatase activity of osteogenic differentiated cells on poly(DTE co 3% PEG-1K carbonate) was higher compared to poly(DTE co 5% PEG-1K carbonate) (Figure 4.20). However, calcium released normalized to cell number is significantly higher on cells on poly(DTE co 5% PEG-1K carbonate) (Figure 4.22).

Evidence from the data presented in this study as well as previous studies, suggests that cell-substrate adhesion is a function of the molar percent of PEG into the polycarbonate backbone. In this study, the relationship between cell-substrate adhesion and motility of undifferentiated hMSCs and OS cells was evaluated. Cell-substrate

adhesion was modified by increasing the molar percent of PEG in the backbone from 3% to 5%. In previous reports, it was shown that there was a biphasic effect of PEG concentration on motility, in that at concentrations of PEG (<3%), there was unsubstantial cell motility, as the concentration of PEG increased to an intermediate concentration (3-4%), motility increased, and then decreased at higher concentrations of PEG (>8%). This effect was observed in the average velocity data at day 11, where OS cells on poly(DTE carbonate) had an unsubstantial average velocity, while cells on poly(DTE co 3% PEG-1K carbonate) had the highest average velocity and cells on poly(DTE co 5% PEG-1K carbonate) had a lower average velocity (Figure 4.39). This suggests that perhaps the proteins adsorbed to the surface of poly(DTE co 3% PEG-1K carbonate) are of an optimum conformation, which may promote cell motility.

The conformation of the adsorbed protein layer was not determined in this study but can be inferred from the thickness of the protein layer, which was determined using the Q-CMD instrument. Serum albumin is a globular protein of dimensions of approximately 8nm x 3.8nm [20]. It is hypothesized that substrates that do not contain PEG have hydrophobic-hydrophobic interactions with proteins which cause the protein to unfold and denature onto the surface. However, substrates containing PEG have hydrophilic-hydrophobic interaction, and results in the stabilization of the protein's globular structure. The average Voight thickness of 10% FBS on poly(DTE carbonate), poly(DTO carbonate), poly(DTE co 3% PEG-1K carbonate), poly(DTE co 5% PEG-1K carbonate), respectively is  $11.46 \pm 4.59$  nm,  $7.04 \pm 3.99$  nm,  $6.64 \pm 4.15$  nm, and  $5.23 \pm 2.36$  nm. An argument could be made that on poly(DTE carbonate), poly(DTO carbonate) and poly(DTE co 3% PEG-1K carbonate) that there are multi-layers of

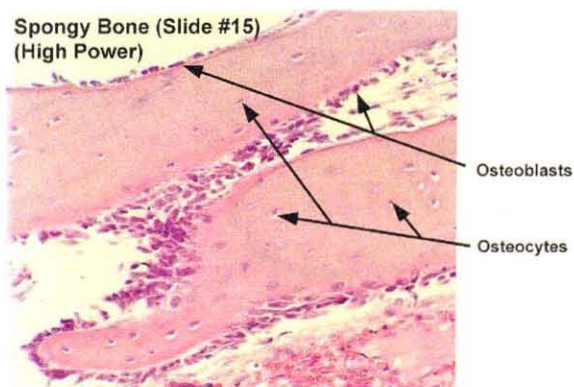


denatured albumin present on the surface, being that the thickness values are greater than 3.8 nm and are almost multiples of 3.8 nm. However on poly(DTE co 5% PEG-1K carbonate), it can be inferred that there is one layer of non-denatured protein. The protein in the denatured state reveals bioactive sites that are recognized by integrins, in which the binding to the protein leads to signal transduction by way of adhesion, differentiation and motility. In order to successfully engraft MSCs onto a substrate, the interface between the cell and substrate, mediated by protein adsorption must be determined.

The work presented in this study is very preliminary and there are many areas where one can expand upon to gain a greater understanding of the cell substrate interface and how it affects cell differentiation. Surface analysis of the thin film substrates in terms of surface topography and residual composition must be analyzed, because it was evident that there was a difference of osteogenic differentiation on spin coated disks and solvent cast films. The surface topography of the spin coated disks was analyzed with SEM (refer to Appendix C), and it was shown to be homogeneous. However this analysis was not conducted on solvent cast films. Furthermore, the protein adsorption experiments must be conducted with extracellular matrix proteins such as fibronectin, vitronectin, and type I collagen which contain the integrin-binding sites: arginine-glycine-aspartate (RGD), that are integral in cell adhesion and osteogenic differentiation. In fact, Salaszyk et al. reported that hMSCs seeded on plates coated with vitronectin or type I collagen in the absence of osteogenic induction medium, can cause differentiation along the osteogenic lineage. Osteogenic differentiation may also be a function of the paracrine factors that cells secrete and may be dependent on cell seeding density. Future studies using various cell seeding densities must be considered. Another concern for future studies is donor

variation. In this study, consistent osteogenic differentiation data was obtained with one donor “Donor 2”. For analysis of different donor types, refer to Appendix B. For future studies, the effect of donor variation must be analyzed. Gene expression, using RT-PCR technology will provide sensitive information about the expression of osteoblast biomarkers.

The aim of this study was to screen tyrosine derived polycarbonates for potential substrates for bone graft scaffolds, which are three dimensional environments. Therefore, understanding the *in vivo* nature of mature osteoblasts is imperative. *In vivo*, osteoblasts exhibit a compact, columnar, apical structure that form a layer of cells at sites of tissue deposition (Figure 5.1)



**Figure 5.1** Histology of cancellous bone of cat. Bone is lined with osteoblasts and osteocytes [6].

This is in contrast to the spread-out morphology of OS cells *in vitro* on the two dimensional polymer substrates: poly(DTE carbonate), poly(DTO carbonate) and poly(DTE co 3% PEG-1K carbonate). OS cells on poly(DTE co 5% PEG-1K carbonate) have a morphology which most resembles mature osteoblasts *in vivo*. Further experiments, on three dimensional scaffolds should be implemented to determine whether

the morphology and biochemical profile of OS cells is differs from the two dimensional environment.

In conclusion, osteogenic differentiation of human mesenchymal stem cells was supported on tyrosine derived polycarbonates in the presence of osteoinductive medium *in vitro*. OS cells on poly(DTE carbonate), poly(DTO carbonate) and poly(DTE co 3% PEG-1K carbonate) are characterized by cell proliferation, the secretion of alkaline phosphatase and calcium, focal adhesion contacts to the substrate and a cuboidal morphology. Moreover, OS cells on poly(DTE co 3% PEG-1K carbonate) is characterized by a relatively high average velocity and random motility coefficient, which is ideal for bone remodeling. The differences in morphology, cytoskeletal organization, cell proliferation and biochemical markers between poly(DTE co 5% PEG-1K carbonate) and poly(DTE carbonate), poly(DTE carbonate) and poly(DTE co 3% PEG-1K carbonate) may be an indication of a highly differentiated osteoblast and further analysis must be made to prove this. These distinct cell responses induced by the polymer substrate could be used in a variety of tissue engineering applications.

## APPENDIX A

### BIOCHEMICAL ASSAYS INCLUDING TCPS SAMPLES

Tissue Culture Polystyrene (TCPS) was used as an internal control for osteogenic differentiation. During the biochemical assays on polymer substrates, hMSCs were seeded onto 96 well TCPS plates (Becton Dickinson: BD 3072, Franklin Lakes, NJ) and 24 well TCPS plates (Becton Dickinson: BD 35 3047, Franklin, NJ).

#### A.1 Cell Proliferation on 96 Well Plates

In this experiment cell proliferation over a 14 day culture was quantified using the DNA assay.

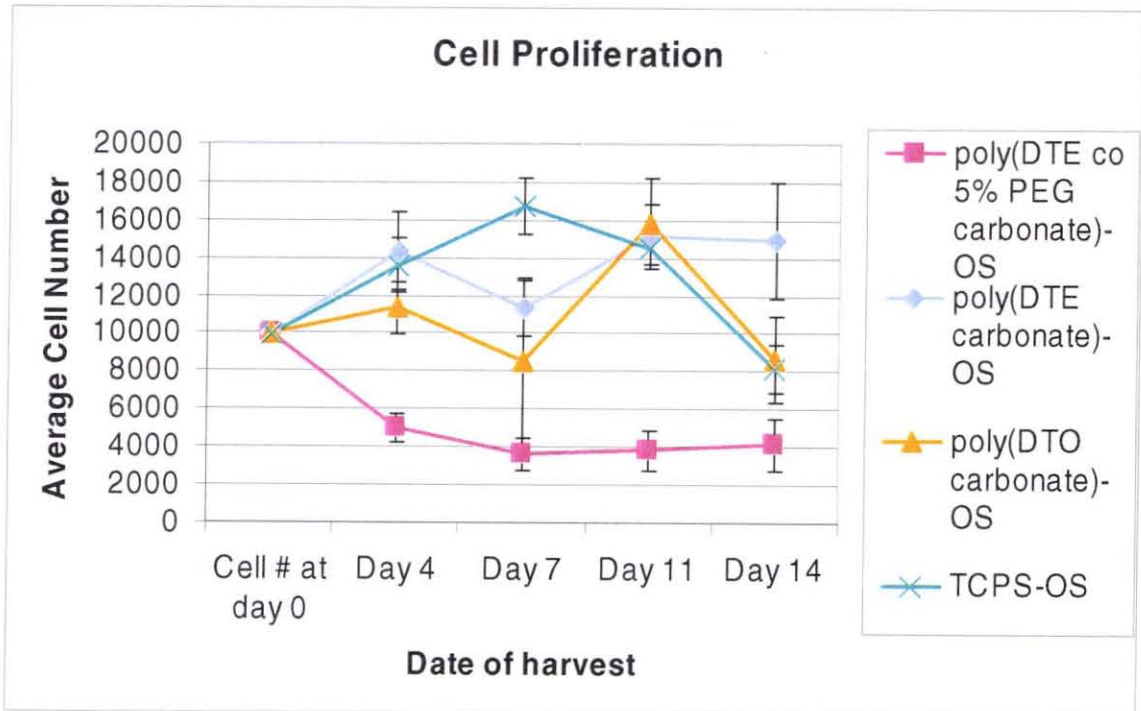


Figure A.1 Cell proliferation of OS cells on polymer substrates.

## A.2 Alkaline Phosphatase Activity on 96 Well Plates

Alkaline Phosphatase, a marker for osteogenic differentiation was quantified.

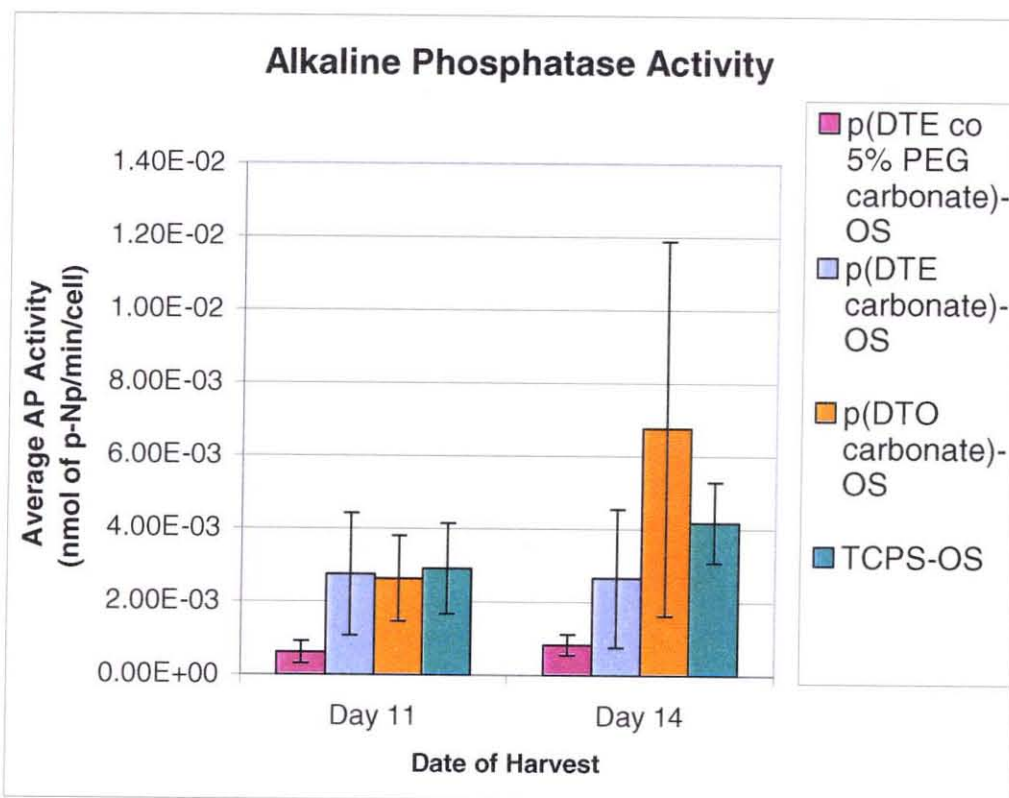
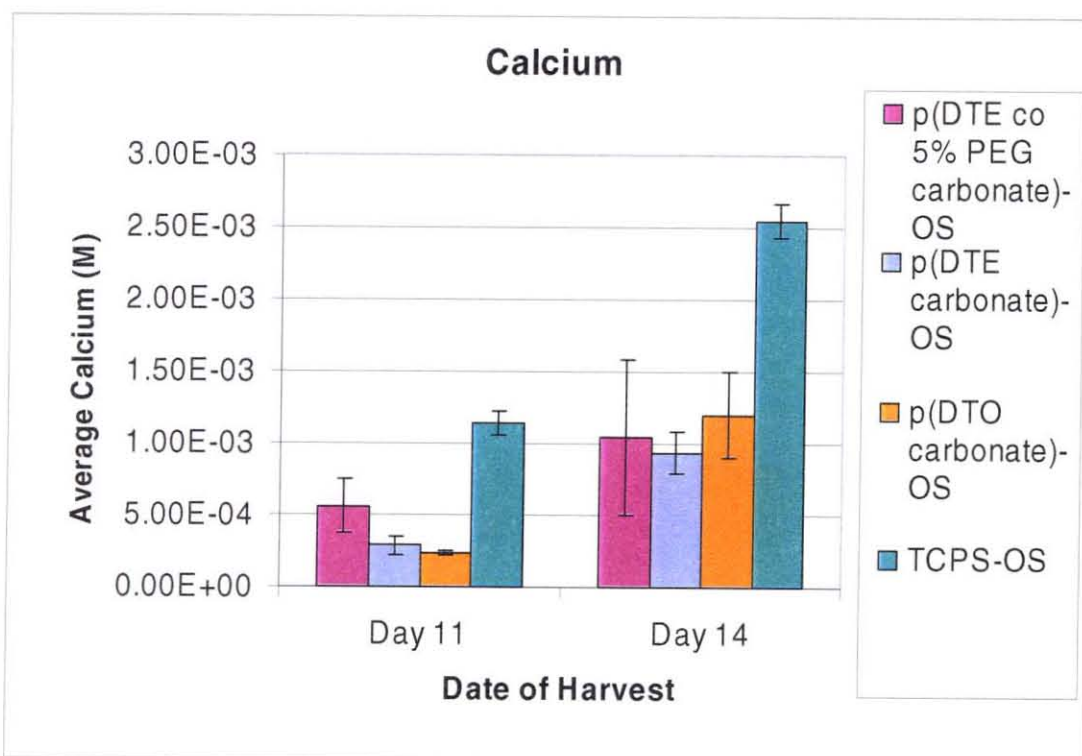


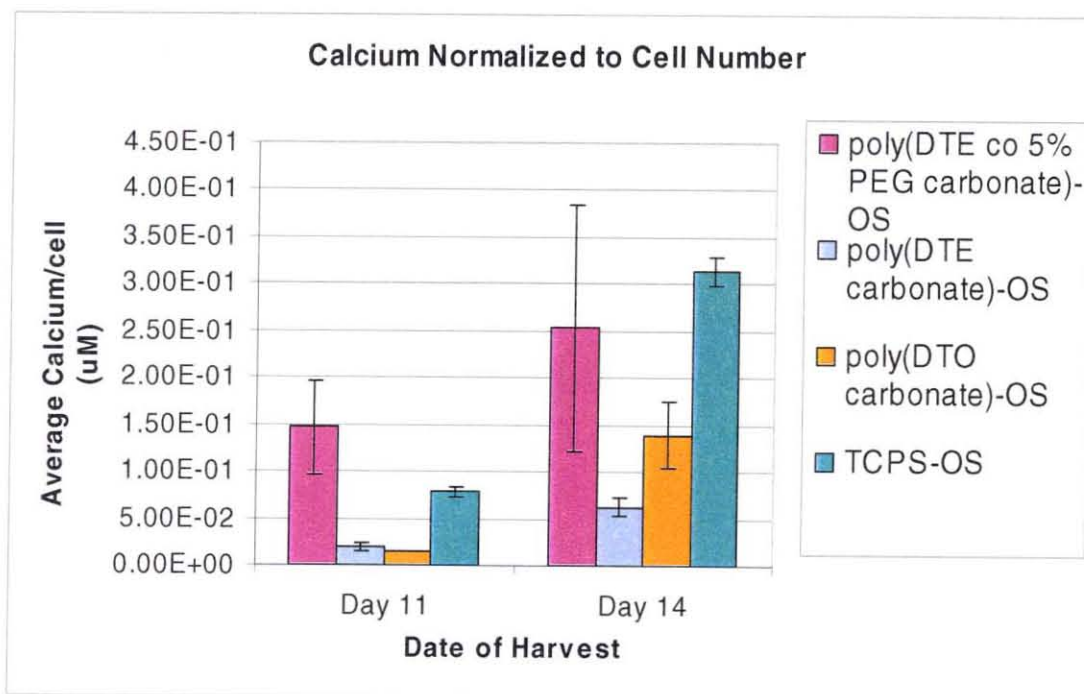
Figure A.2 Alkaline Phosphatase activity of OS cells on polymer substrates.

### A.3 Calcium Production on 96 Well Plates

Calcium production, a marker for osteogenic differentiation was quantified.



**Figure A.3** Calcium production of OS cells on polymer substrates.



**Figure A.4** Calcium normalized to cell number, of OS cells on polymer substrates.

#### A.4 Cell Proliferation on 24 Well Plates

In this experiment cell proliferation over a 14 day culture was quantified using the DNA assay.

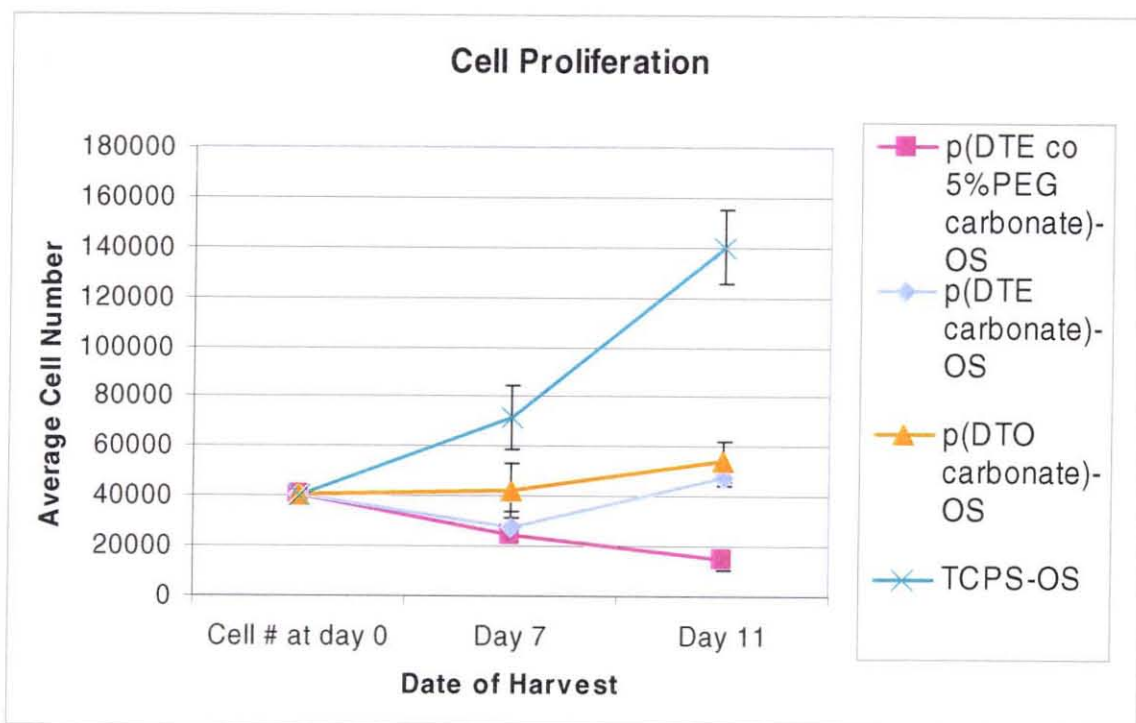
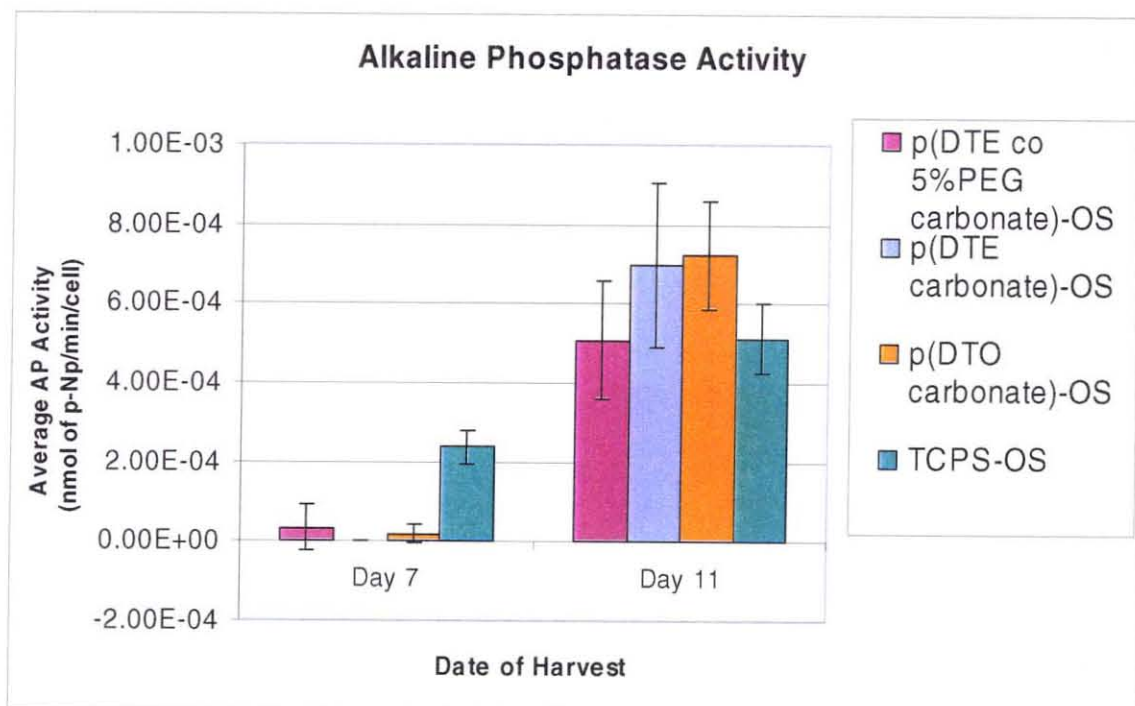


Figure A.5 Cell proliferation of OS cells on polymer substrates.

### A.5 Alkaline Phosphatase Activity on 24 Well Plates

Alkaline Phosphatase, a marker for osteogenic differentiation was quantified.

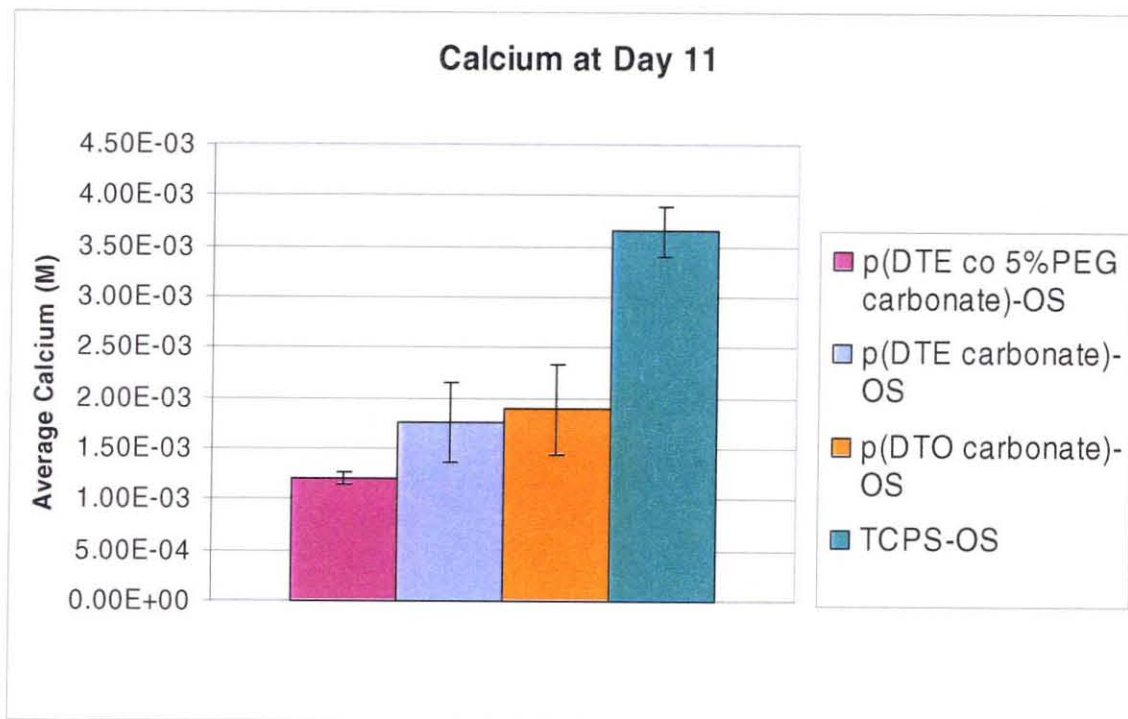


**Figure A.6** Alkaline Phosphatase activity of OS cells on polymer substrates.

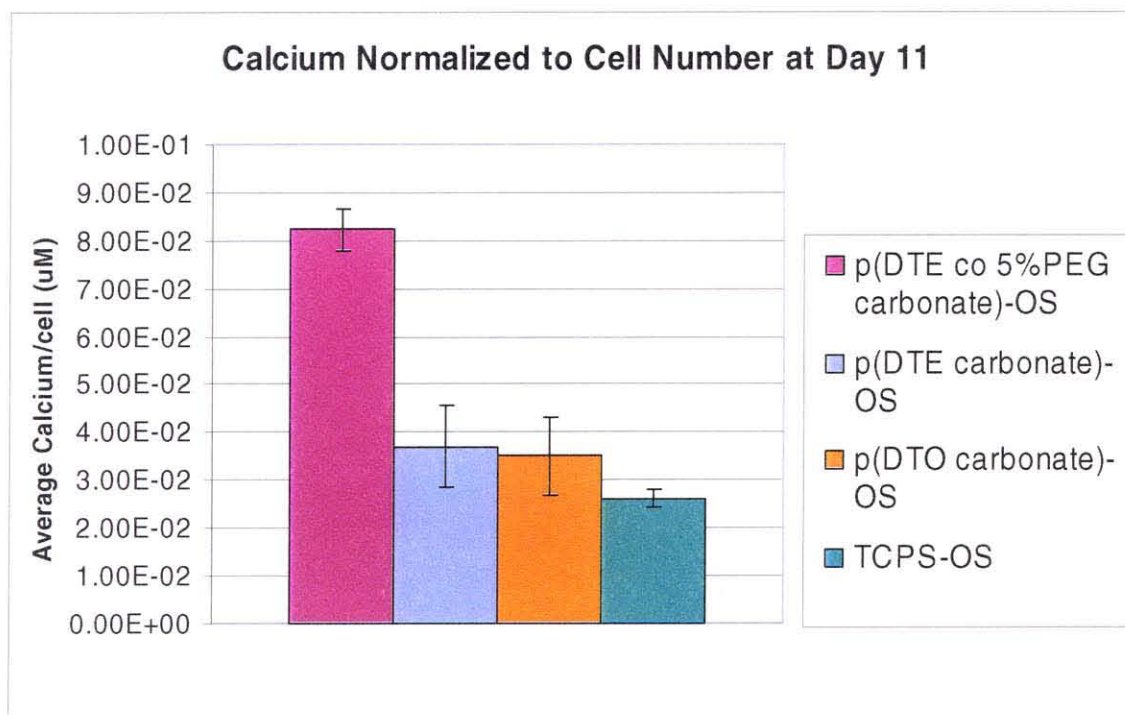


### A.6 Calcium on 24 Well Plates

Calcium production, a marker for osteogenic differentiation was quantified.



**Figure A.7** Calcium production of OS cell on polymer substrates.



**Figure A.8** Calcium normalized to cell number, of OS cells on polymer substrates.

### A.7 Osteocalcin

Osteocalcin is a marker for mature osteoblast differentiation[5]. The amount of osteocalcin present in the extracellular matrix of the samples was determined using the Intact Human Osteocalcin EIA kit (Biomedical Technologies, Stoughton, MA). In this EIA kit, the provided standards and samples were quantified for osteocalcin by detecting the monoclonal antibody that is specific to it. A microplate coated with monoclonal antibody was treated with the standards and samples and osteocalcin antiserum for 2 and ½ hours at 37°C. The plate was washed with phosphate-saline buffer 2-3 times before adding streptavidin-horseradish peroxidase for 30 minutes. The plate was washed again and a solution of hydrogen peroxide and TMB solution added to the plate, which was incubated for 10 minutes in the dark. The stop solution was added to the wells of the plate and the resulting absorbance was read at 450 nm on the absorbance plate reader (Biotek).

### A.7.1 Osteocalcin Results

At day 11, osteocalcin production on osteogenic differentiated hMSCs (OS) is statistically lower compared to the other substrates. Osteocalcin production was normalized to average cell number. There was no statistical difference between the substrates when normalized to cell number. However, osteocalcin production normalized to cell number of the substrates is statistically higher than osteocalcin production normalized to cell number of tissue culture polystyrene.

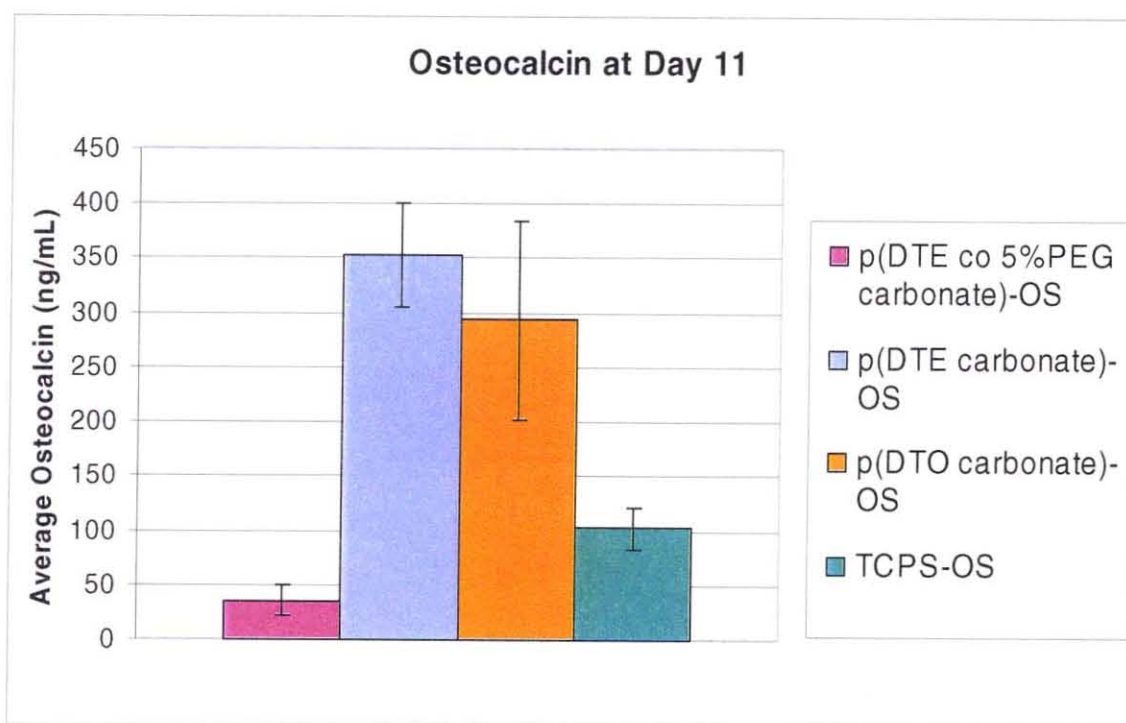
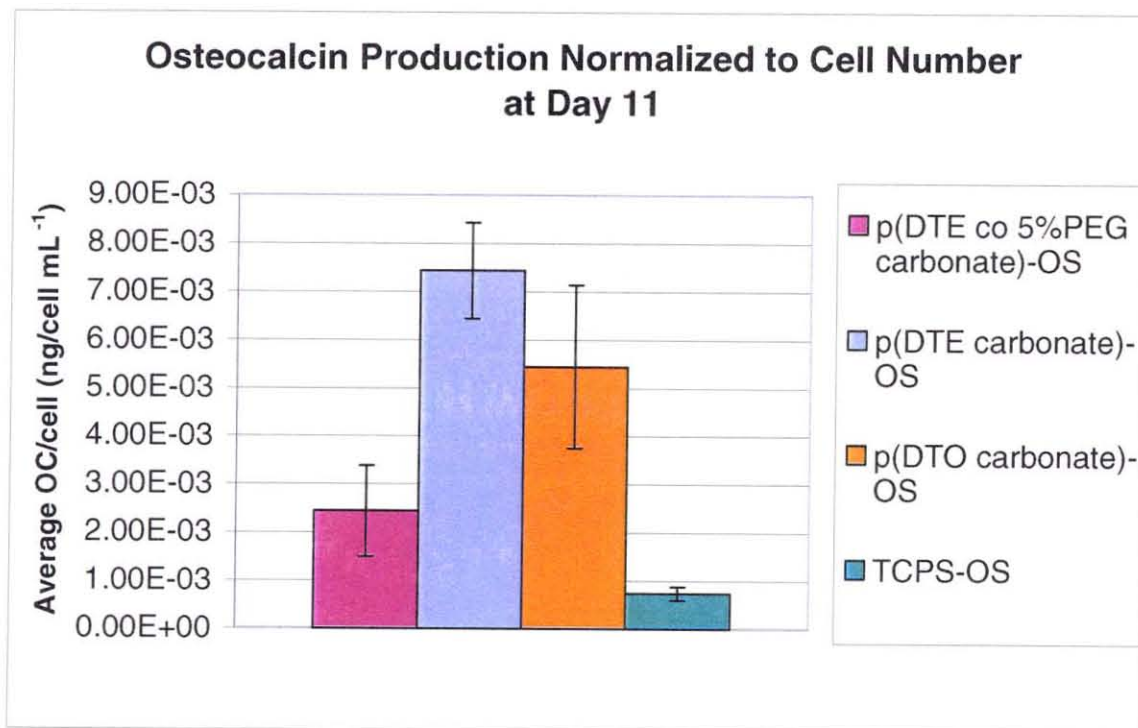


Figure A.9 Osteocalcin production of OS cells on polymer substrates.

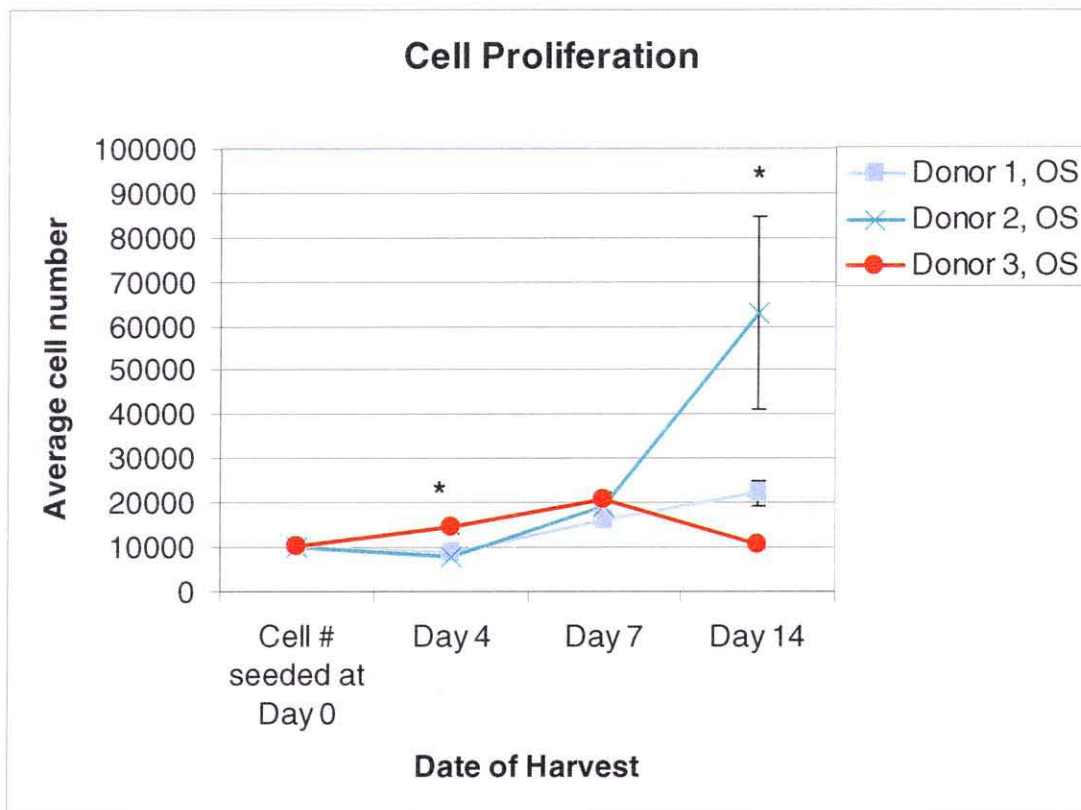


**Figure A.10** Osteocalcin normalized to cell number, of OS cells on polymer substrates.

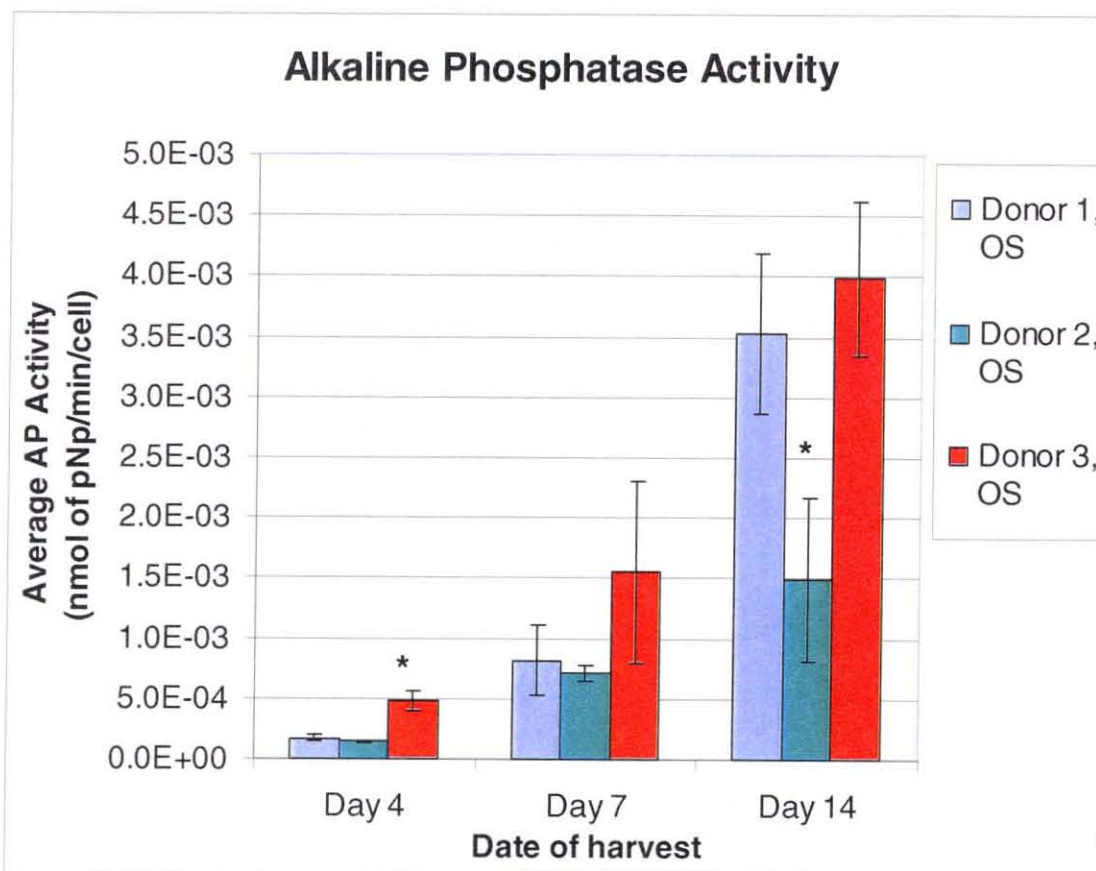
## APPENDIX B

### DONOR VARIABILITY

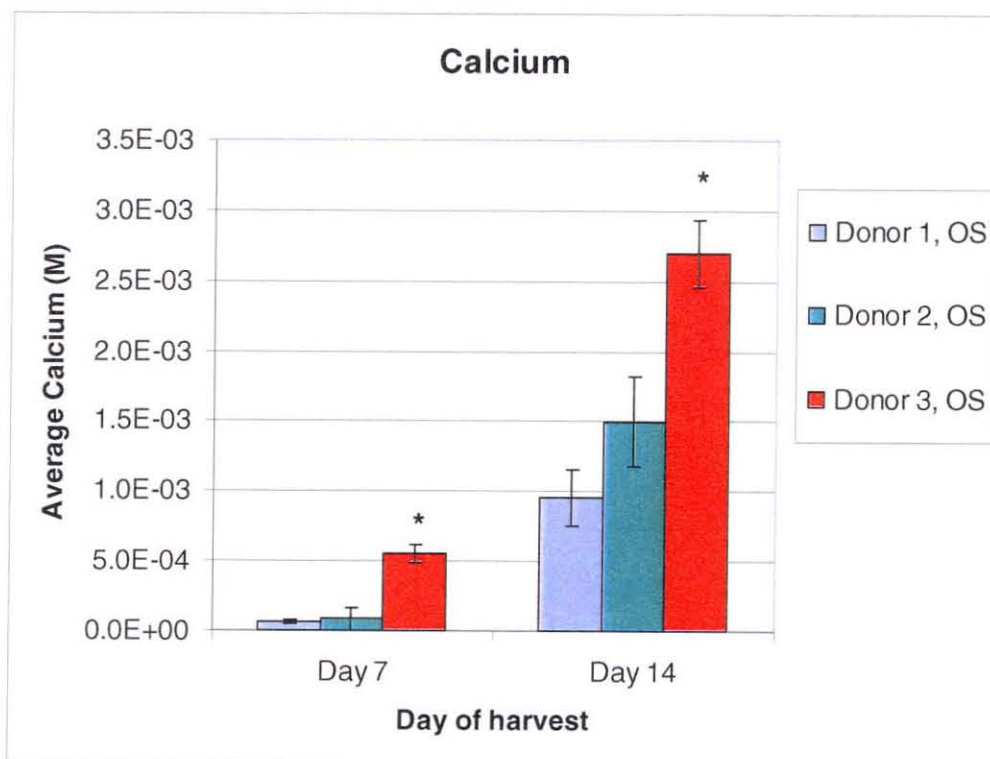
Donor variation may have an effect on osteogenic differentiation, which was first evidenced by Phinney et al. In an experiment conducted in May 2005, donor variability was observed in cell proliferation and osteogenic differentiation assays on 96 well tissue culture polystyrene (TCPS) plates. In this experiment, at day 14, it is evident that Donor 3 has the highest calcium production and Alkaline Phosphatase activity, but the lowest cell number. Donors 1 and 2 have similar response at day 7 but not at day 14. It is not known whether, the variability is inherent in the donors.



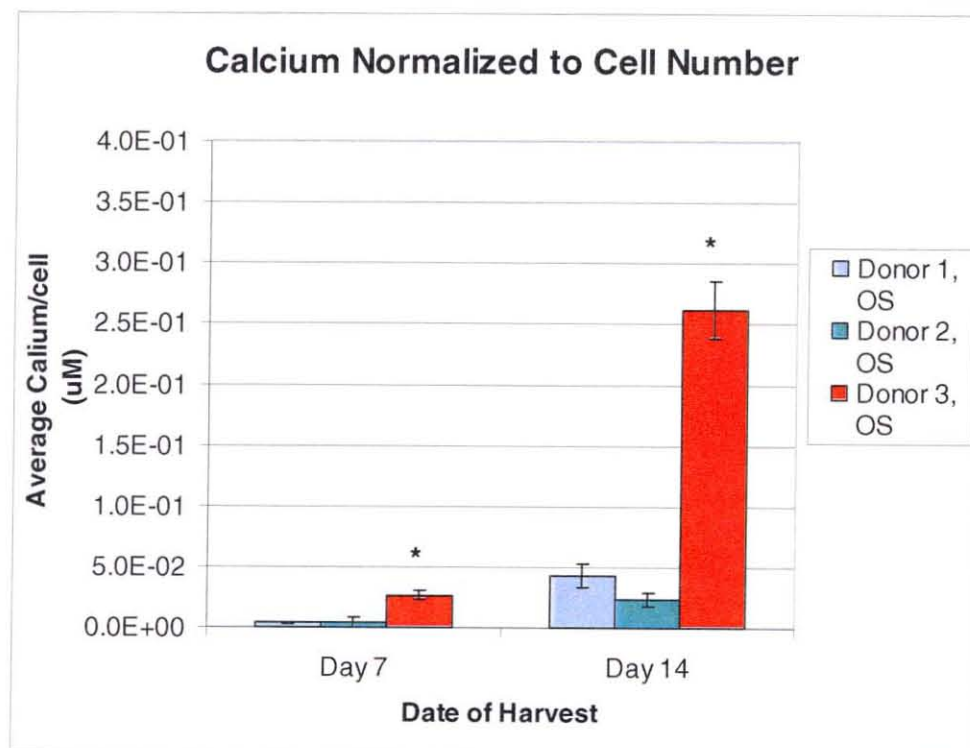
**Figure B.1** Cell proliferation of OS cells of Donors 1-3 on TCPS. Asterisks at day 4 indicates statistical difference, ( $p < 0.05$ ) between Donor 3 to both Donors 1 and 2. Asterisks at day 14 indicates statistical difference between Donor 2 to Donors 2 and 3.



**Figure B.2** Alkaline Phosphatase activity of OS cells of Donors 1-3 on TCPS. Asterisks at day 4, indicate statistical difference ( $p < 0.05$ ), between Donor 3 to both Donor 1 and 2. Asterisks at day 14, indicate statistical difference ( $p < 0.05$ ) between Donor 2 to both Donor 2 and 3.



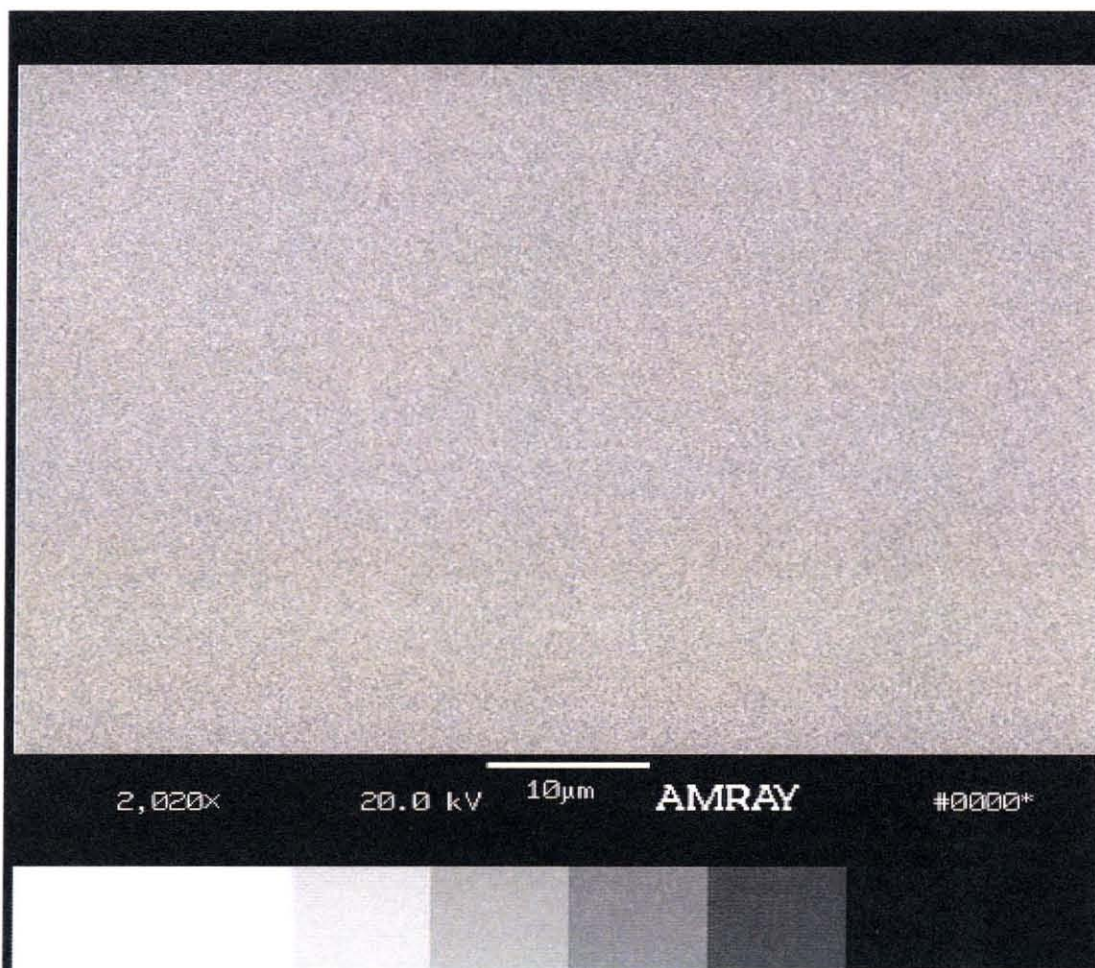
**Figure B.3** Calcium production of OS cells of Donors 1-3 on TCPS. Asterisks indicate statistical difference ( $p < 0.05$ ), between Donor 3 to both Donors 1 and 2.



**Figure B.4** Calcium normalized to cell number of OS cells of Donors 1-3 on TCPS. Asterisks indicate statistical difference ( $p < 0.05$ ), between Donor 3 to both Donor 1 and 2.

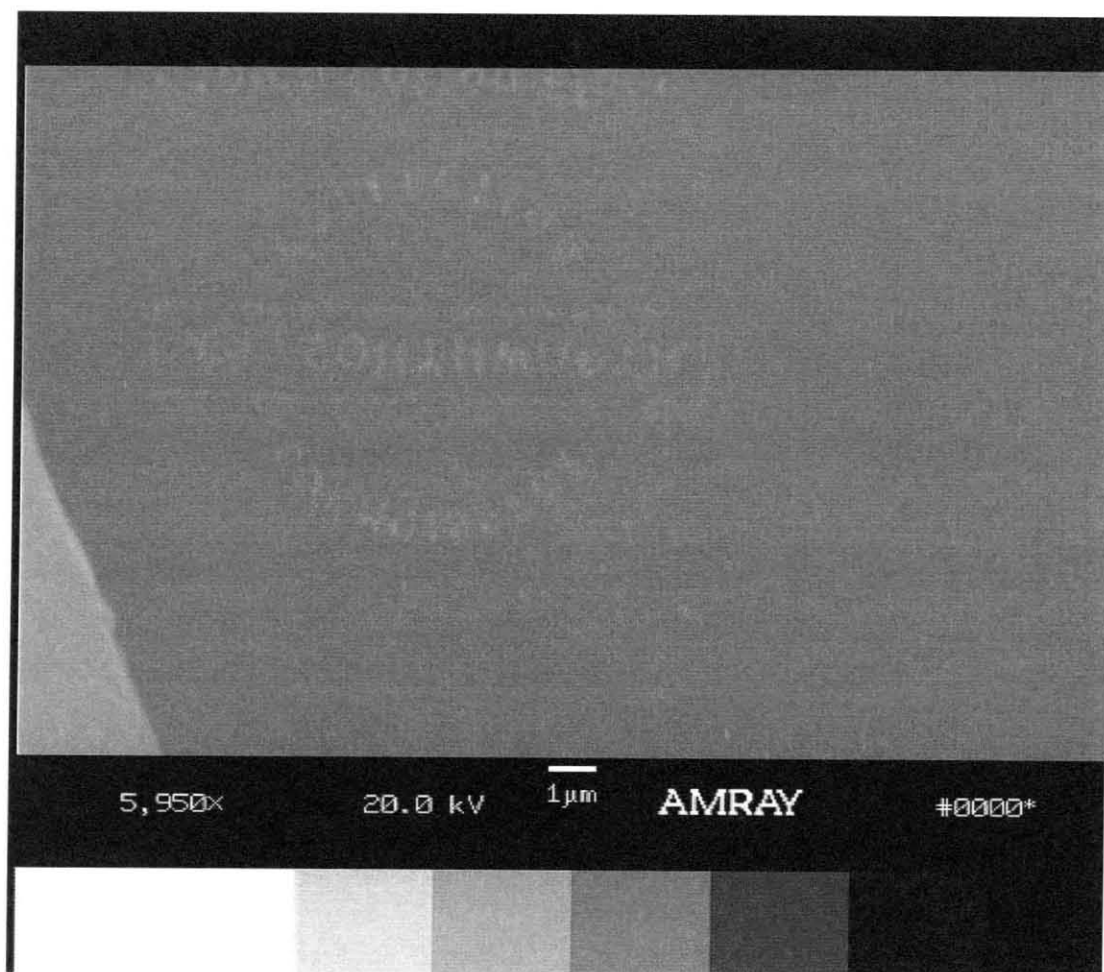
**APPENDIX C**  
**SEM IMAGING**

The integrity of the spin coated surface was observed with Scanning Electron Microscopy (SEM). The following SEM images are of spin coated disks prepared in November 2005. The homogeneity of the surface indicated a smooth surface. SEM was not conducted on solvent cast films.



**Figure C.1** SEM image of poly(DTE carbonate) at 2,020x.





**Figure C.2** SEM image of poly(DTE co 5% PEG-1K carbonate) at 5,950X.

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