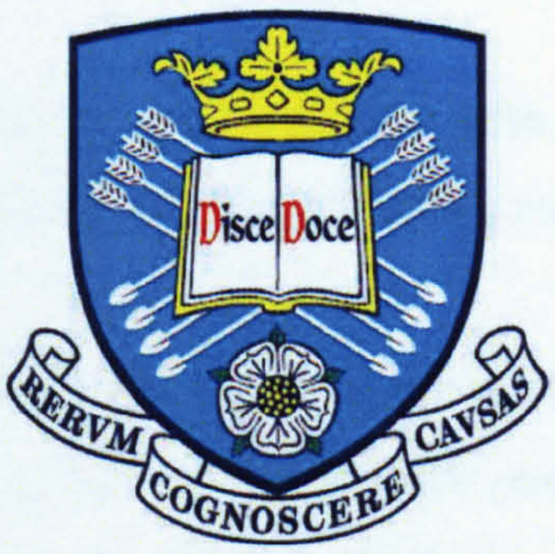
The Effects of Substrate Stiffness on Mesenchymal Stem Cell Proliferation and Differentiation



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Abbreviations

%	percentage
γ	gamma
α	alpha
β	beta
°C	degrees centigrade
µg	microgram
µl	microlitre
µM	micromolar
2°	secondary
AFM	atomic force microscopy
ALP	alkaline phosphatase
ANOVA	analysis of variance
BMSC	bone marrow stromal cells
Ca^{2+}	calcium
CCD	charge-coupled device
CD	cluster of differentiation
CFU-f	colony forming unit-fibroblastic
cm	centimetre
cm^2	square centimetre
cm^3	cubed centimetre
CO_2	carbon dioxide
COL	collagen
CPS	counts per second
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E	modulus
ECM	extracellular matrix
EDTA	ethylenediaminotetraacetic

Ev	electronic volts
FACS	fluorescein activated cell sorting
FAD	flavin adenine dinucleotide
FITC	fluorescein isothiocyanate
g	gram
H_2O	water
HCl	hydrochloric acid
Hz	hertz
IgG	immunoglobin G
IBMX	isobutylmethylxanthine
IMS	industrial methylated spirit
KDa	kilodaltons
KPa	kilopascal
L	litre
LVDT	linear variable displacement transducer
M	molar
Mag	magnification
MAPC	multipotent adult progenitor cells
mbar	millibar
mg	milligram
Mg^{2+}	magnesium
Min	minute
ml	millilitre
mM	millimolar
mm	millimetre
MPa	millipascal
MRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
N	number
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
nm	nanometre
nM	nanomolar

NMM	non-muscle myosin
OC	osteocalcin
OD	optical density
ON	osteonectin
p	probability
Pa	pascal
PA	polyacrylamide
PBS	phosphate-buffered saline
PCA	personal cell analyser
PDMS	polydimethylsiloxane
PE	phycoerythrin
pH	potential of hydrogen
PI	propidium iodide
PM^2	photomultiplier 2
p-NP	para-nitrophenol
p-NPP	para-nitrophenolphosphate
PTFE	polytetrafluoroethylene
RhoA	Ras homolog gene family (member A)
RNase	ribonuclease
ROCK	Rho kinase
Rpm	revolutions per minute
SD	standard deviation
Si	silicon
SS	serum supreme
STP	standard temperature and pressure
TCP	tissue culture plastic
Tris	tris(hydroxymethyl)methylamine
VSMC	vascular smooth muscle cell
V / v	volume per volume
W	watt
W / v	weight per volume
XPS	x-ray photoelectron spectroscopy

Abstract

The aim of the project was to test the hypothesis that substrate stiffness will affect MSC proliferation and differentiation. In order to achieve this two systems were developed; a natural fibrin substrate, whereby altering the concentration of fibrinogen changes the stiffness of the resultant gel and an artificial PDMS substrate where the stiffness is controlled by altering the degree of crosslinking.

To determine the effect of various fibrin matrices, providing more physiological growth conditions, on the MSC phenotype; cells were cultured on the gels and then analysed or re-plated onto TCP before analysis. It was found that cells, that had an initial 7-day culture period on the fibrin, proliferated and maintained their osteogenic differential potential better when compared to cells pre-cultured on TCP. Similarly, a concentration relationship between colony number and fibrin concentration was seen with a decrease in colony number as the fibrin number increased suggesting that progenitor cell numbers are better maintained on low-stiffness gels. Furthermore, direct culture on the gels demonstrated a stiffness related increase in colony number.

PDMS is easily produced with a large range of mechanical properties. Uncoated PDMS does not support MSC attachment and growth *in vitro* and therefore an acrylic acid coating was applied. Although XPS analysis was unable to establish that a complete coating was deposited on all of the substrates, once coated the PDMS supported MSC attachment and growth. CFU-f efficiency was not directly altered by the mechanical properties of the underlying substrate, however, the differentiation of the cells showed a trend; with an increase in osteoblastic differentiation as the stiffness increased. This trend was also seen under high-density culture conditions with no correlation to the rate of proliferation.

Although the exact mechanism is unknown the data presented here supports the concept that substrate signals influence MSC growth and differentiation.

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CHAPTER ONE: Literature Review

1.1 Tissue engineering & stem cells

Langer and Vacanti, (1993) stated that " Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function". To reach this common goal, three strategies were defined; 1) create engineered matrices and introduce them into the body at the repair site, 2) introduce cells alone to the desired site or 3) combine the two and introduce an engineered matrix with cells seeded within it (Fuchs et al., 2001). The latter two methods are the most common but for success in tissue engineering applications large numbers of cells are needed. Recently, tissue engineering techniques have been applied in the generation of bladders for the treatment of cystoplasty. In this pioneering study, 700 x 10^6 of both autologous urothelial and muscle cells were seeded onto collagen-polyglycolic acid scaffolds demonstrating the need for large numbers of cells in the successful engineering of human organs (Atala et al., 2006). The cells, within the tissues of the adult organism, follow a specific lineage undergoing proliferation, migration, differentiation and maturation. Cells derived from adult tissues have a limited proliferation capacity but the earliest cell in the lineage is the stem cell. A stem cell can be defined as a cell that is capable of self- renewal (ability to produce an identical daughter cell) but can also differentiate to various mature progeny (Zipori, 2004), making it the ideal choice for use in tissue engineering.

Embryonic stem cells, derived from the inner cell mass of the developing embryo have the potential to develop into any cell type of the adult organism and propagate indefinitely (Thomson et al., 1998). However, research into these cells and their potential in a therapeutic setting has led to great controversy both morally and ethically and has led to the search for an alternative cell population (Frankel, 2000). Adult stem cells, although offering a more restricted differentiation potential, also show a self-renewable phenotype enabling expansion in culture to create the large numbers needed. A number of so-called 'adult stem cells' have been defined including haematopoietic, intestinal, neural, mesenchymal and epithelial (Atala et al., 2006). It was once considered that these particular stem cells were destined to become mature cells of the tissue in which they reside, but now with the demonstration that the cells are not as differentially restricted as originally thought they have received a renewed interest (Petersen et al., 1999) (Pittenger et al., 1999).

1.2 Mesenchymal stem cells

The bone marrow consists of three cellular systems; haematopoietic, endothelial and stromal (Deans and Moseley, 2000). The stromal system comprises the supporting connective tissue of the haematopoietic system and within it a heterogeneous population of cells exist including adipocytes, osteogenic cells, smooth muscle cells, reticular cells, macrophages and endothelial cells (Short et al., 2003). The bone marrow is also home to two populations of stem cells the haematopoietic stem cells (capable of repopulating the circulating blood system) and mesenchymal stem cells.

It was the pioneering work of Friedenstein, Owen and co-workers who helped define MSC by showing the growth of colonies from cells isolated from the bone marrow stroma of postnatal and adult organisms. The cells were described as fibroblastic, rapidly adherent, non-phagocytic and clonogenic with a capability of extensive proliferation *in vitro* and in a series of publications their multipotency was demonstrated both *in vivo* and *in vitro* (Owen and Friedenstein, 1988).

Since their first description and original name of colony forming unit fibroblasts (CFU-F) many names have been used to describe the cells. The earlier names have now been replaced with marrow stromal cells (MSC), mesenchymal stem cells (MSC) or mesenchymal progenitor cells (MPC) (Minguell et al., 2001). To avoid any ambiguity the term mesenchymal stem cell (MSC) will be used to refer to the bone marrow derived progenitor cells used experimentally within this report.

Currently, the bone marrow stroma is commonly considered to be the most enriched source of MSC. Isolation is typically from the superior iliac crest of the pelvis in humans and larger animals whereas MSC are generally harvested from the mid-diaphysis of the tibia or femur in rodents. Although the bone marrow offers an easy source for isolation, MSC are found in numerous locations within the adult organism including adipose tissue (Zuk et al., 2001), dermis (Young et al., 2001) and blood (Zvaifler et al., 2000). It has been postulated that, due to the wide distribution of MSC, the bone marrow stroma is a common pool of multipotent cells that gain access to various tissues via the circulation (Tuan et al., 2003a), with the suggestion that mobilisation occurs in response to wound signals or disease conditions (Barry and Murphy, 2004). At the present time, with a lack of a unifying definition, this is difficult

to prove but will no doubt become clearer when specific markers defining the cells are characterised.

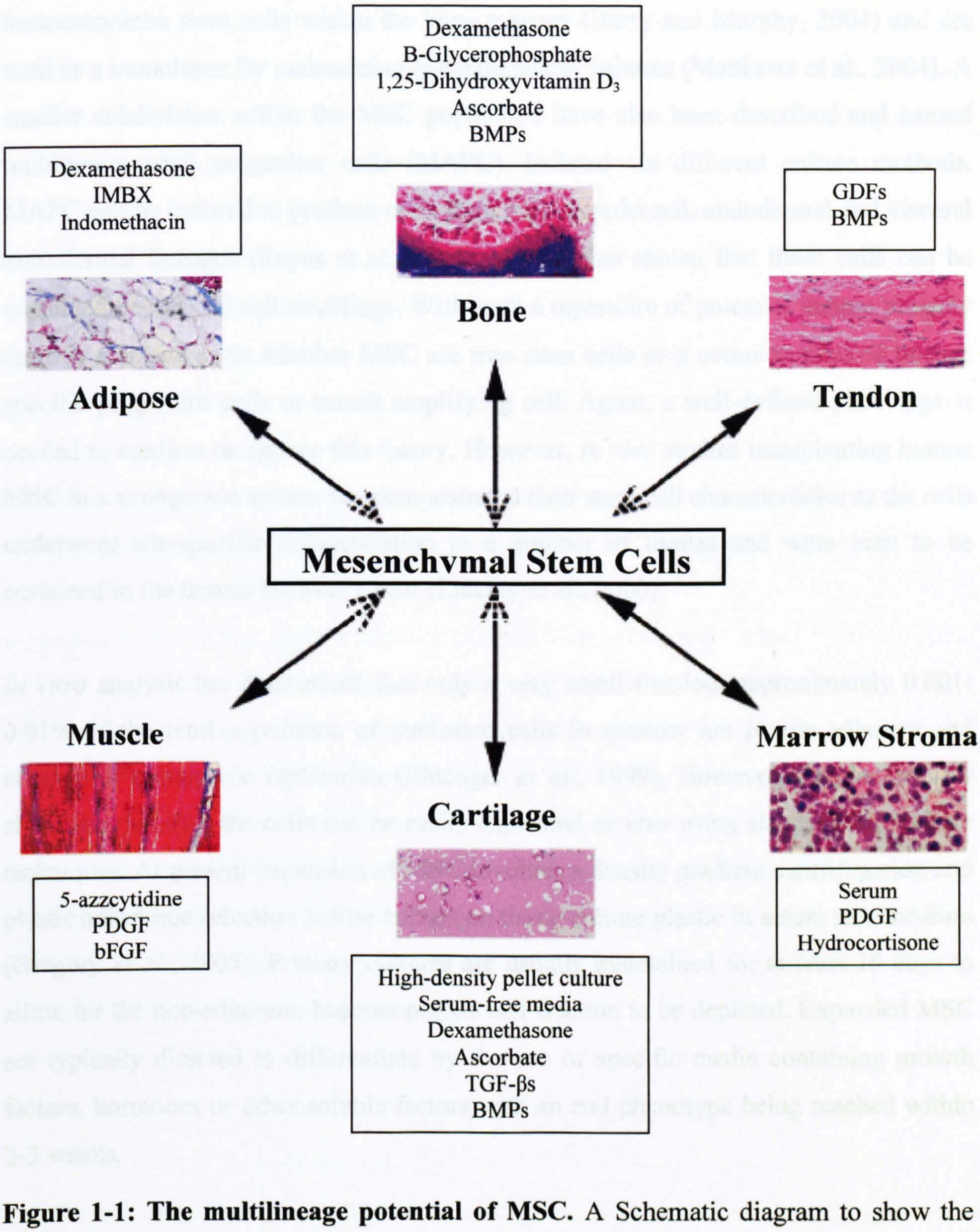


Figure 1-1: The multilineage potential of MSC. A Schematic diagram to show the multilineage potential of MSC to a number of different tissue types with reference to the *in vitro* culture conditions needed to induce the chosen route of differentiation (adapted from Tuan *et al.*, 2003b).

MSC are defined as multipotent due to their potential for multilineage differentiation. Even as clonally isolated cells, they exhibit the ability to differentiate down a number of

connective tissue lineages (**Figure 1-1**) including bone, adipose tissue, cartilage, tendon and muscle (Pittenger et al., 1999). MSC also provide the stromal support system for haematopoietic stem cells within the bone marrow (Barry and Murphy, 2004) and are used as a monolayer for maintaining haematopoietic cultures (Matthews et al., 2004). A smaller subdivision within the MSC population have also been described and named multipotent adult progenitor cells (MAPC). Isolated via different culture methods, MAPC can be induced to produce cells from neuroectodermal, endodermal and visceral mesodermal lineages (Reyes et al., 2001). It was also shown that these cells can be expanded beyond 50 cell doublings. With such a repertoire of potential mature progeny there is a debate as to whether MSC are true stem cells or a common pool of lineage specific progenitor cells or transit amplifying cell. Again, a well-defined phenotype is needed to confirm or oppose this theory. However, *in vivo* studies transplanting human MSC in a xenogeneic system has demonstrated their stem cell characteristics as the cells underwent site-specific differentiation in a number of tissues and were seen to be sustained in the tissues for over a year (Liechty et al., 2000).

In vitro analysis has determined that only a very small fraction, approximately 0.001-0.01% of the total population of nucleated cells in marrow are plastic adherent and capable of clonogenic replication (Pittenger *et al.*, 1999). However, despite the low abundance *in vivo,* the cells can be easily expanded *ex vivo* using standard cell culture techniques. At present expansion of MSC involves a density gradient centrifugation and plastic adherence selection before culture on tissue culture plastic in serum rich medium (Gregory *et al.*, 2005). Primary cultures are usually maintained for at least 10 days to allow for the non-adherent, haematopoietic cell fraction to be depleted. Expanded MSC are typically directed to differentiate by the use of specific media containing growth factors, hormones or other soluble factors with an end phenotype being reached within 2-3 weeks.

By light microscope, *in vitro* culture expanded MSC look fibroblastic, exhibiting mostly fusiform and cuboidal morphology (Kassem, 2004). In culture the growth pattern of early passaged cells, at a low seeding density is typically a prolonged lag phase of 72-96 hours followed by a stage of rapid growth when the doubling time can be as low as every 10 hours. The proliferation is then seen to plateau as colonies size increases (Prockop *et al.*, 2003). This pattern is seen to be repeated for four to five cycles if the

cells are serially passaged at low seeding densities. Numerous reports claim that populations of MSC are heterogeneous when characterised both morphological and by surface epitope expression (Mets and Verdonk, 1981) (Colter *et al.*, 2000). Classification of the heterogeneous populations emphasize the presence of large flat cells that have been referred to as both 'type II' cells and 'mMSC' and a smaller population of smaller, agranular cells called 'type I' or 'RS-1' cells (Mets and Verdonk, 1981) (Colter *et al.*, 2001). Furthermore, description of the RS-1 cells giving rise to a further population of small, granular cells (RS-2 cells) has been made. mMSC are characterised as slowly proliferating, mature cells whilst the RS cells are a smaller population of rapidly self-renewing cells that possess a higher level of multipotency (Colter *et al.*, 2000).

Osteogenic differentiation is achieved when expanded MSC are cultured with a glucorticoid (commonly the synthetic glucocorticoid dexamethasone) and ascorbate in serum rich medium. When cultured in a monolayer, with the addition of these supplements the cells acquire a cuboidal osteoblastic morphology with the upregulation of ALP expression. ALP, a cell surface protein, is widely accepted as a marker of new bone formation. Deposition of a calcium rich mineralised extra cellular matrix is also observed (Barry and Murphy, 2004). Phenotypic expression at different stages of differentiation is summarised in **Figure 1-2**, which shows schematically that the cells progress through three stages; proliferation, maturation and mineralisation overtime in culture, with proliferation decreasing as the cells mature (Stein and Lian, 1993). Although inductive agents are known the exact molecular regulation is currently unknown and is an active area of investigation.

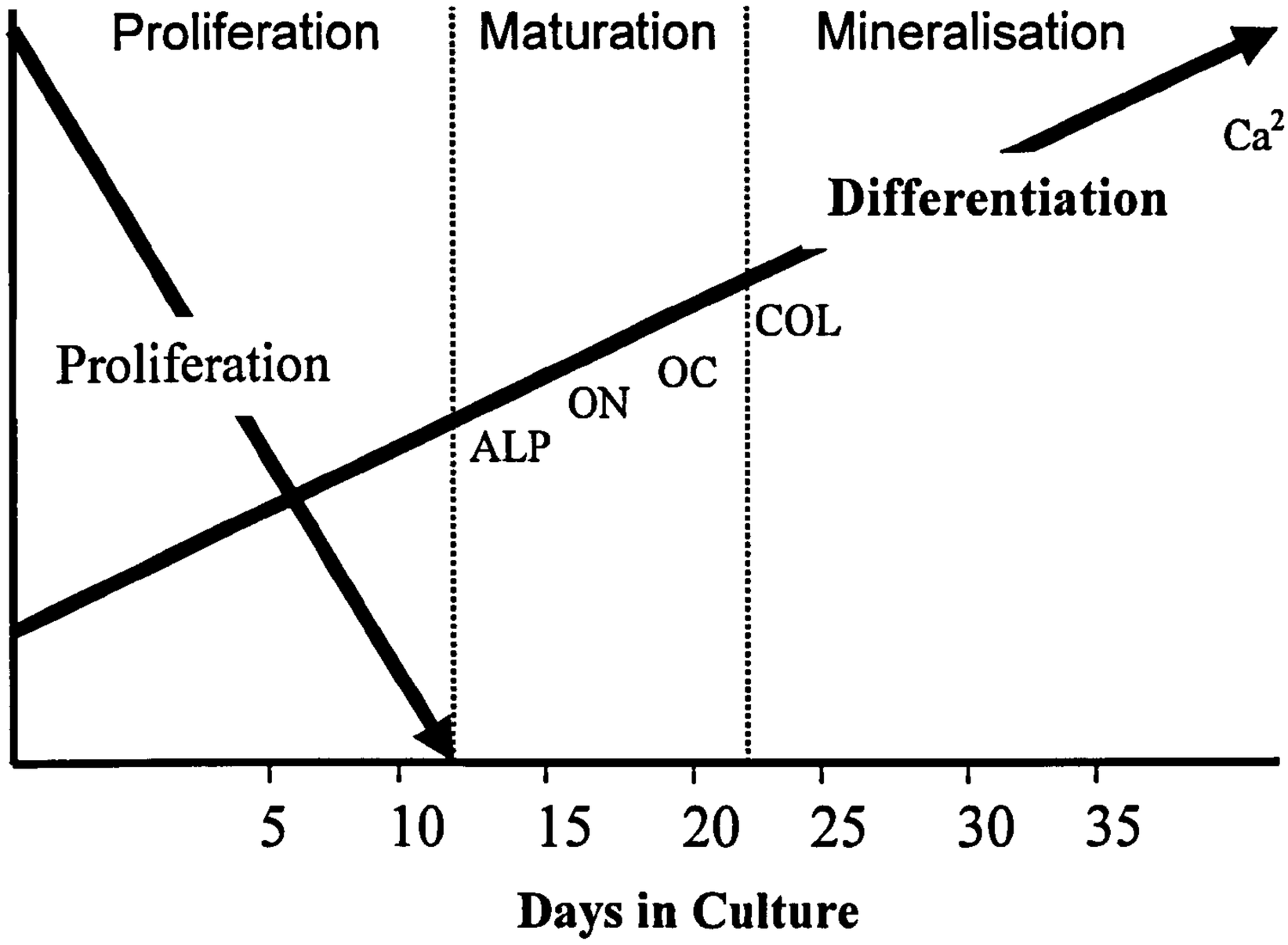


Figure 1-2: Schematic diagram to illustrate the relationship between cell growth and the differentiated gene expression of osteogenic cells. The cell progresses through three stages; proliferation, maturation and then mineralisation in the transformation from an undifferentiated cell to a cell of the osteogenic lineage. OC=osteocalcin, ON= osteonectin, Col= Collagen, ALP= alkaline phosphatase (adapted from Stein and Lian, 1993).

Although MSC are controversially defined as stem cells, they do not exhibit properties of immortalised cells and demonstrate a finite lifespan with reports of human MSC reaching a maximum of only 38 ± 4 population doublings (Bruder *et al.*, 1997). The limited life span is due to telomere shortening (Banfi *et al.*, 2002) and the accumulation of senescent cells (DiGirolamo *et al.*, 1999). Growth of the cells also correlates to the age of the patient, site of extraction and disease state (Stenderup *et al.*, 2003). Impairment of the differentiation potential is also seen over time in culture (DiGirolamo et al., 1999).

At present, there are no specific surface markers to define MSC; their phenotypic characterisation is usually defined by their multipotent differentiation potential. Extensive research has been done to try and identify specific markers to aid the isolation of MSC from bone marrow cells and antibodies have been identified to react with the non-haematopoietic progenitors bone marrow stromal cells however, none have gained a wide acceptance. Simmons and Torok-Storb., (1991) were the first to provide positive identification of MSC via the STRO-1 antibody and used it to isolate a more homogenous population of MSC. Van Vlasselaer et al., (1994) identified anti Sca-1, a monoclonal antibody that selects for an enriched population of osteoprogenitor cells from whole bone marrow. Regardless of the lack of unifying consensus on a specific marker, immunophenotyping is important in the identification of MSC and often a combination of a number of surface antigens are used for the enrichment of the most homologous population. It is generally accepted that MSC are positive for THY-1 (CD90), vascular cell adhesion molecule-1 (CD106) and hyaluronate receptor (CD44) and distinct from haematopoietic stem cells by being negative for CD45, CD41, CD14, CD34 and T or B cell markers (Alhadlaq and Mao, 2004). Consensus on which markers are most useful for the isolation and enrichment of MSC will enable better extrapolation of results and will allow for better comparison of research completed by different groups.

1.3 The role of MSC in the clinical setting

MSC hold a lot of potential for use in clinical applications. They have attracted attention as a potential source of autologous cells (thereby avoiding an immune response) (Prockop et al., 2003), that are obtainable from a clinically accessible site. Preliminary research has also demonstrated that allogenic transplantation may be possible due to the cells hypo-immunogenic nature and the fact that no tumorgenicity is seen; a problem predicted to be encountered when using ES cells (Gruen and Grabel, 2006). Liechty et al., (2000) were the first to introduce and incorporate MSC into an animal model and showed that transplanted human cells were capable of site specific differentiation into a number of tissue lineages including adipocytes, chondrocytes and bone marrow stromal cells. This study helped to reveal the fate of transplanted MSC and highlighted the potential use of the cells in a number of clinical applications. Three main applications have been highlighted for the potential use of the cells; local implantation, systemic transplantation and tissue engineering.

1.3.1 Local implantation

Ex vivo expansion followed by the implantation to localised sites could see MSC being used to treat local diseases including coronary artery disease, non-healed chronic skin wounds and fracture healing. Promising results in animal models have demonstrated the potential use of MSC in Parkinson's disease (Schwarz et al., 1999), stroke (Li et al., 2002), and myelin deficiency (Jin et al., 2002). Potentially the cells could also be used in the treatment of hepatic failure (Beck et al., 2005), myocardial infarction (Orlic *et al.*, 2001) and many neurological conditions (Mezey et al., 2000, Kopen et al., 1999). Early studies in a porcine model of myocardial infarction demonstrated that MSC grafted into the damage area were capable of engraftment into the myocardium host. Furthermore, expression of muscle specific markers and an improvement in contractile function holds promise for the use of MSC in cardiomyoplasty to reduce the pathological effects caused by myocardium infarction (Yang et al., 2001).

1.3.2 Systemic transplantation

With the success of systemic transplantation of haematopoietic stem cells, it was inevitable that research would be carried out on the use of allogenic MSC as a transplantation therapy. Horwitz *et al* (1999), transplanted allogenic bone marrow for the treatment of osteogenesis imperfecta (type 1 collagen defect) and although a clinical

improvement was seen the results remain controversial as no direct evidence that the cells formed either bone or collagen was seen (Dominici et al., 2001). Clinical trials have also been completed using MSC in the treatment of Hurler's syndrome and Metachromatic Leukodystrophy and although the research remained inconclusive in the treatment of the diseases the study demonstrated that donor allogeneic MSC infusion is viable and safe (Koc et al., 2002).

Genetic modification of the stem cells could also lead to the combination of stem cell therapy with gene therapy (Kassem, 2004). Animal studies employing genetically modified bone marrow stromal cells were locally implanted into a rat model of Parkinson disease and have shown some promise for the genetic modification of MSC in the treatment of neurological diseases (Schwarz *et al.*, 1999). Once again it is the cells ability to replicate for long periods of time that makes them an excellent target.

1.3.3 Tissue engineering

MSC hold a lot of promise for use within tissue engineering. Their proliferative capacity enables them to be expanded greatly *ex vivo* and then after combining with biomaterials, for structural support, implanted to the desired site. Addition of growth factors or other soluble factors to the scaffold could induce differentiation to the needed cell type. Bruder *et al.*, (1998) used MSC grown on a scaffold to repair a femur defect in a canine model, highlighting the potential use of MSC in treating large fractures Quarto *et al.*, (2001) improved bone repair with the use of autologous MSC mixed with hydroxyapatite scaffolds in a cell-based tissue-engineering approach to treat three patients with large bone defects. Although bone and cartilage are the most studied tissues for use of MSC in tissue engineering, animal experiments have also been conducted looking at tendon and muscle repair (Bagnaninchi *et al.*, 2006, Krampera *et al.*, 2006). To date the majority of research has been completed in animal models with fewer studies making it into the clinical setting.

Use of MSC in therapeutic applications is still in the very early stages. For MSC to be employed successfully in any clinical application, it is essential that the cells are first properly identified, methodologies for their expansion and differentiation clearly defined with maintenance of the exact phenotype ensured throughout.

1.4 Modulation of differentiation

No simple set of master genes or mechanisms have been identified for the induction of the MSC down all of the different differentiation pathways (Dennis and Charbord, 2002) making it difficult to identify the inductive and repressive factors that are responsible for directing or inhibiting differentiation and phenotypic expression of the cells. It is now thought that *in vivo* lineage progression of MSC is regulated by the interaction of many extrinsic cues with the intrinsic cues dictated by the genomic potential of the cell (Mwale *et al.*, 2006). The extrinsic cues, including tissue damage, chemotaxins, multipotentiality, proliferation and interaction with other cells, have been well recognised in the past (Otto and Rao, 2004).

A variety of chemical stimuli are used for the *in vitro* differentiation of MSC to a number of different cell types. The most common soluble factors added are highlighted in (Figure 1-1). As already mentioned, the addition of dexamethasone, ascorbic acid and β-glycerophosphate induces osteogenesis *in vitro* but is unlikely to represent the highly complex variety of physiological inducers present *in vivo* (Gregory *et al.*, 2005). *In vitro,* differences in serum batches alone are enough to alter the differentiation of CFU-f cultures and therefore has led researches to determine the effects of different mitogens on the growth and differentiation of CFU-f's in a controlled environment. An ever-growing list of chemical inducers including hormones and growth factors and their effects (both as stimulators and inhibitors) on MSC have been previously described (Minguell et al., 2001).

The effects of mechanical signals on the cell are also very important but their effects on differentiation have somewhat been overlooked until recently. A number of stresses are placed upon the cell including fluid sheer stress, elongational and pressure stresses and can be generated both internally, within the cell or externally by for instance gravity, muscle contraction, blood flow or contact with a solid (Georges and Janmey, 2005). A summary of recent *in vitro* research looking into the biophysical effects on cells is given below. References to work completed on stem cells *in vitro* is given wherever possible.

1.4.1 Mechanical stress

It is well established that mechanical loading is important to the homeostasis of a number of connective tissues, but the effects it has on differentiation are unclear. One

system where research is in progress to determine if there is an effect is in the production of cartilage. Placing chick limb bud cells under dynamic mechanical stress has been shown to influence the regulation of chondrogenesis with increases in cartilage nodule and [^{35}S] sulphate incorporation (Elder et al., 2000) and an increase in collagen production (type II and X) and aggrecans (Takahashi et al., 1998). The effect on the differentiation of the cells was seen to be dose dependant with the magnitude of stress applied directly proportional to the production of collagen type (II), another well-defined marker of chondrogenic differentiation and a linear increase in chondrogenesis in response to an increase in cyclic frequency via cartilage nodule density and ^{35}S incorporation (Takahashi et al., 1998).

1.4.2 Cyclic stretch

In vitro work looking at cyclic stretch (biaxial tensile loading) was influenced by the observations that unloading of bones *in vivo* lead to decreased bone mass density (Estes et al., 2004). Thomas and El Haj., 1996). hypothesised that this effect would also be seen *in vitro* with bone marrow cells responding to changes in mechanical strain. On flexible substrates cyclic biaxial stretch caused an increase in ALP and was significantly larger than the control when 230 μstrain was added, an effect hypothesised to be mediated via prostaglandins. Simmons et al., (2003a) confirmed this result by also positively linking cyclic stain to MSC differentiation by investigating the effect of equibiaxial cyclic strain (3%, 0.25 Hz) substrate deformation on the proliferation and osteogenic differentiation of MSC. It was found that application of the strain inhibited proliferation and stimulated a 2.3-fold increase in matrix mineralisation compared to unstrained cells. Recently these observations have been taken to the next stage with the first report of MSC differentiating down the osteogenic lineage under cyclic strain alone. The cells placed within a 3D collagen construct showed a significant increase in BMP-2 mRNA expression levels strained at 10 % when compared to unstrained controls at both 7 and 14 day time points (Sumanasinghe et al., 2006).

1.4.3 Multiaxial loading

Multiaxial loading has been used *in vitro* to represent the complex loading seen by ligament *in vivo.* In response to the multidimensional mechanical strains (translational and rotational strain), isolated progenitor cells were shown to upregulate a number of

known ligament tissue markers including collagen type III and tenascin-C (Altman et al., 2002).

1.4.4 Shear stress

Shear stress has been shown to influence differentiation of stem cells in a number of different systems. Wang et al., (2005) showed that sheer stress significantly induced the mature endothelial cell markers in a murine embryonic mesenchymal progenitor cell line model with an increase in such markers as CD31 and von Willebrand factor at both the mRNA and protein levels. Work completed on human MSC in 3D constructs has shown that shear stress can effect the proliferation, CFU-f formation and differentiation potential of the cells when cultured within a perfusion bioreactor system (Zhao et al., 2007).

Recently, different aspects of the local microenvironment surrounding the cell has been shown to influence many attributes of cellular function, however, it is only recently that the effects on differentiation have been demonstrated.

1.5 Substrate properties

1.5.1 Surface chemistry

It has been known for some time that subtle changes in surface chemistry can directly influence cellular response (Boyan et al., 1996). Typically an effect is seen due to differences in the adsorption of exogenous proteins that thereby alters the attachment of the cell and subsequently leads to differences in morphology and growth. Differences in cell adhesion can also influence the rate of cell division (Danen and Yamada, 2001).

Even though a lot of research has been completed looking at the effect of surface chemistry on cellular behaviour, it has only recently been appreciated that surface chemistry may directly influence stem cell differentiation. Different chemistries have been defined that both enhance and suppress differentiation, but currently it remains unclear as to whether this is a direct effect or whether the chemistry is affecting the cell's ability to lay down a collagenous ECM (a pre-requisite for differentiation) or simply the cell's attachment and subsequent proliferation (Mwale et al., 2006) (Curran et al., 2005).

Although it is important to consider the effect on the surface chemistry, for the purpose of this study we are aiming to keep the chemistry constant whilst looking at the effects of surface stiffness on MSC growth.

1.5.2 Topography

It is well appreciated that topography of the underlying substrate will influence a number of cellular responses (Curtis and Wilkinson, 1997), with topographical features in the nanometer range known to influence cell behaviour (Dalby et al., 2004). Culturing osteoblasts on micro-rough surfaces has been shown to enhance osteoblastic differentiation (Boyan et al., 2003) and recent studies have shown that the combination of nanotechnology with biochemical cues enhances the neuronal differentiation of MSC (Yim et al., 2007).

1.5.3 Hydration

Although essentially liked to the chemistry of the substrate surface, both hydrophobicity and hydrophilicity have been shown to affect cellular behaviour typically by altering the initial attachment of the cell (van Kooten et al., 1992). Hydrophilic materials favour the

retention of native protein confirmation and enables a stability that is often lost when a protein irreversibly binds to a hydrophobic surface (Underwood et al., 1993). Research in the past has demonstrated that a more hydrophilic surface enhances cell proliferation, but differentiation may also be enhanced on hydrophilic surfaces (Godbole, 2004).

When trying to create a substrate that can be modified to produce a range of mechanical properties (i.e. stiffnesses) it is important to note that when one variable is changed this often leads to a number of other variables also being changed. It is a challenge to minimise the other variables but also to ensure that they are considered when any conclusions are being reached.

1.6 Substrate mechanics

1.6.1 Fibroblasts

It was the revolutionary work of Harris et al., (1980) who first employed an artificial substratum to study cellular behaviour. By observing and measuring wrinkling patterns created by cell traction they were able to calculate the absolute magnitude of the shear forces exerted by individual fibroblasts. However, it was not until 1997 that Pelham and Wang were the first to report an affects of substrate stiffness on cell behaviour, by actually changing the mechanical properties of the underlying cell culture substratum. Using polyacrylamide gels, they devised a novel method of coating the otherwise chemically inert polymer with collagen and subsequently looked at the adhesion and locomotion of 3T3 fibroblastic cells and normal rat kidney epithelial cells. A compliance dependent change in both the motility and adhesion was seen with cells on the softer substrates showing reduced spreading and increased motility. This initial paper on the effects of substrate stiffness on cells has lead to much research in the field and is summarised below.

After the innovative study on substrate stiffness, Wang et al., (2000) suggested that the flexibility of the underlying substrate would not only affect adhesion and motility but could also regulate the growth and apoptosis of cells. NIH 3T3 cells cultured on soft, flexible substrates showed a decrease in the rate of DNA synthesis, coupled with an increase in the rate of apoptosis. This effect was seen for normal but not transformed cells leading to the suggestion that a mechanism is in place to regulate cell shape, growth and survival on properly defined substrates, a mechanism lost in cancerous cells where unregulated growth of transformed cells is observed.

Although traction force mapping of cells is a relatively old method, the advent of the PA culture substratum revolutionised the technique. Improved optical quality and the use of fluorescent beads modernised the older wrinkle method previously preformed on silicone substrates. Work completed on fibroblasts on the PA gels showed the cells apparent preference for a stiffer substrate. The cells were recorded migrating from soft to stiff regions, with an increase in traction forces and spread area. This phenomenon termed "durotaxis" where cells will move to stiffer region is very important in tissue engineering design. Tissue engineered scaffolds not made uniformly or produced using a number of materials could see cells migrating to an undesired area. However, it could

be used to an advantage by using the phenomenon to seed cells in a particular location (Lo et al., 2000). This work completed on the artificial substrates confirmed research preformed by Sheetz et al., (1998) who speculated that movement of a cell can be directed by the stiffness of the ECM, which acts to create an environmental cue.

Soft lithography, microfabrication on elastomeric membranes can create micrometer-scale patterns of proteins on surfaces (Chen et al., 2003). Ostuni et al., (2000) suggested that cell shape can subsequently effect the degree of spreading and migration with the work confirming the hypothesis that there is a link between cell shape and behaviour (Ingber, 1990). Further work employing the technique has also showed that cell spreading and the geometric shape of the attached area can also regulate cell behaviour (Parker et al., 2002) (Brock et al., 2003) and that the shape and size of the cell can determine its passage through the cell cycle (Huang et al., 1998). More recently, McBeath et al., (2004) have shown that cell shape effects the differentiation of MSC with a more rounded cell phenotype differentiating into adipocytes and a more spread shape favouring an osteoblastic phenotype. Whilst an important tool in the study of the effect of cell shape and substrate mechanics, soft lithography technique relies on differences in ECM density, a factor which could influence cellular behaviour, and often the investigation is of isolated cells alone.

Most cell types are generally not viable when in suspension and are therefore said to be anchorage dependant. Adherent cells together with the ECM exist to provide a relatively elastic microenvironment, yet *in vitro* cells are attached and cultured on TCP, a very hard substrate (Discher et al., 2005). TCP, whist a convenient culture substrate, is of limited physiological relevance. Research has shown that on stiffer surfaces upregulation of the cytoskeleton and the number of cell-matrix adhesions is seen. This is due to the cell taking on a more spread morphology typically with long, straight actin bundles stretching across the cytoplasm. In contrast cells on softer gels are observed to be more spherical where the f-actin is more diffuse and localisation is predominately beneath the cell membrane (similar to non-adherent cells) (Pelham and Wang, 1997) (Engler et al., 2004a). Grinnell, (2000) showed that the morphology of fibroblasts imbedded within collagen gels is clearly distinct from those cultured on TCP, with research showing that culture on TCP also induces the formation of stress fibres (Georges and Janmey, 2005).

The work described so far has been predominately completed using fibroblasts, the effect of substrate stiffness on morphology does however appear to be cell type specific.

1.6.2 Endothelial cells

Research completed on endothelial cells cultured on both collagen gels (Vernon et al., 1992) and fibrin gels (Vailhe et al., 1997) has shown an increase in network like structures on softer gels. Gels made softer by decreasing the ECM concentration enabled the cells to form long capillary-like tube structures. Consistent with these findings, human umbilical vein derived endothelial cells were more elongated and exhibited a interconnected network on softer collagen gels compared to a stiffer substrate (Sieminski et al., 2004).

1.6.3 Neurones and Glial cells

Balgude et al., (2001) found the rate of neurite extension to be inversely correlated to the mechanical stiffness of agarose gels with neurons grown on softer substrates forming more than three times as many branches as those grown on stiffer gels. Flanagan *et al*, (2002) extended this work by allowing mouse spinal cord primary neurons to grow and extend neurites for several weeks in culture on Matrigel-coated polyacrylamide gels with varying deformability. They verified that neurons grew well on the gels and preferentially on the softer gels.

1.6.4 Myocytes

It has been hypothesised that substrate stiffness will have a great effect on myocytes function and behaviour as coupling of a contracting cell to the substrate is fundamental for their phenotype. Myotubes ultimately transmit actomyosin contractions through their attachments to the matrix. On a stiff substrate these contractions will be isometric whilst softer substrates allow a contraction to occur (Engler et al., 2004b). Similar to results seen for fibroblasts and endothelial cells, myoblasts plated on polyacrylamide gels showed a correlation that the spread area of the cell increased as a function of the substrate elastic modulus. However, this change was observed to be time dependant and after 24 hours the cells were seen to spread on both soft and stiff substrates.

Most cells types, studied so far, seem to be affected by substrate stiffness. The general trend is that cells adhere and spread better on stiffer matrices with the exception of

endothelial cells and neurones that prefer a softer matrix. This contrast in behaviour could be related to their native tissue and the substrate interactions and fits the hypothesis that MSC are located within a common pool that travel away from their niche to a new microenvironment in response to tissue damage (Katayama et al., 2006). Also fitting with this theory is the report that MSC have extreme sensitivity to tissue level elasticity and are capable of differentiating in response to it to the neurogenic, myogenic and osteogenic lineages (Engler et al., 2006). The trend observed is not only important in tissue engineering but also in understanding disease processes, morphogenesis and tissue repair strategies (Discher et al., 2005). Caution must also be taken when using MSC in the clinical setting. For example, in the use of the cells in the treatment of myocardial infarction, cells cannot just be injected into the desired location if stiffness will affect cellular behaviour, and in particularly the differentiation of the stems cells.

Despite this knowledge, the majority of *in vitro* research focuses on the addition of soluble factors (growth factors and cytokines) to drive the cell towards a particular fate. With the majority of cell culture completed on TCP there is a high possibility that the effect of the microenvironment on the behaviour of the cells *in vitro* will lead to results being interpreted inaccurately especially if the effect seen has an additive or synergistic effect with the biochemistry used (Estes et al., 2004).

1.7 Mechanotransduction

Although limited, the research completed so far strongly suggests that cells can sense their local microenvironment and feel the stiffness of the underlying substrate, responding to it by altering their attachment, shape, mobility etc. Although the exact mechanism as to "how the cell senses the change in mechanical property of the substrate and in turn transduce this in to a biological response?" is unclear, many theories have been postulated. It is also a challenge to assess what is "real" to the cell when most of the hypotheses proposed are based on research completed on TCP a very none physiological substrate.

Lo et al., (2000) proposed one theory that cells can sense their local microenvironment by exerting probing forces and then directly sensing the distance of receptor movement or by monitoring the magnitude of counter-forces after the exertion of a given amount of energy. This hypothesis has been extended further with scientists believing that the mechanical feedback could lead to activation of stress-sensitive ion channels (Lee et al., 1999) or activation of tension-sensitive proteins (Lo et al., 2000). Pelham and Wang, (1997) hypothesised that a cell interprets the underlying material by direct sensing through anchored integrin receptors which subsequently leads to changes in tyrosine phosphorylation and myosin-generated cortical forces

Although the exact mechanism is unclear, it is accepted that it is through mechanotransduction. Mechanotransduction is the process by which cells convert mechanical energy into electrical or biochemical signals and is a well-established mechanism in a number of other systems (LeMasurier and Gillespie, 2005). The cell attachments to the matrix via transmembrane proteins from the integrin family, intercellularly this link is continuous with the actin cytoskeleton via cytoplasmic plaques comprised of a number of proteins including talin, α-actinin, filamin and tensin (Schwarz and Bischofs, 2005). It is through the actions of drawing on these adhesions and by gauging the amount of resistance that it is hypothesised that a cell could sense the compliance of the substrate. A stiff surface will resist the pull whereas a soft surface will not.

A number of observations have been made to support the idea of mechanotransduction with strong evidence pointing to the involvement of the cytoskeleton in the process.

Cells are very malleable and can reorganise their cytoskeleton in response to external mechanical signals. A number of cell types are seen to have a more spread morphology on stiffer substrates with the recruitment of actin fibres. Choquet et al., (1997), also determined that cells were able to respond to a force by strengthening integrin-mediated contacts a phenomenon they coined mechanotaxis. This finding is supported by the fact that cells cultured on gels down regulate the number of proteins in focal adhesion complexes (Marganski et al., 2003); and alone is evidence that the mechanical property of the gel is enough to influence protein expression.

Other support for the hypothesis includes the observation that when cells are cultured after treatment with cytochalasin D, a fungal metabolite that disrupts the actin cytoskeleton, measurable traction forces disappear (Georges and Janmey, 2005). This strongly suggests that the process is F-actin dependant. It has also been reported that focal adhesion kinase-null fibroblasts are unable to mechanosense (Georges and Janmey, 2005). Studies have also shown that the disruption of microtubules via the actions of Nocodazole has no effect and would therefore suggest that they are not significantly involved in the process.

Myosin inhibitors have also been used in the investigation of the possible role of cell contractibility in the sensing of substrate compliance. Blebbistatin a potent non-muscle myosin II inhibitor and Y-27632 a ROCK inhibitor have both been used to demonstrate that cell shape, cytoskeletal tension and RhoA signalling are all involved (McBeath et al., 2004).

1.8 Tensegrity model

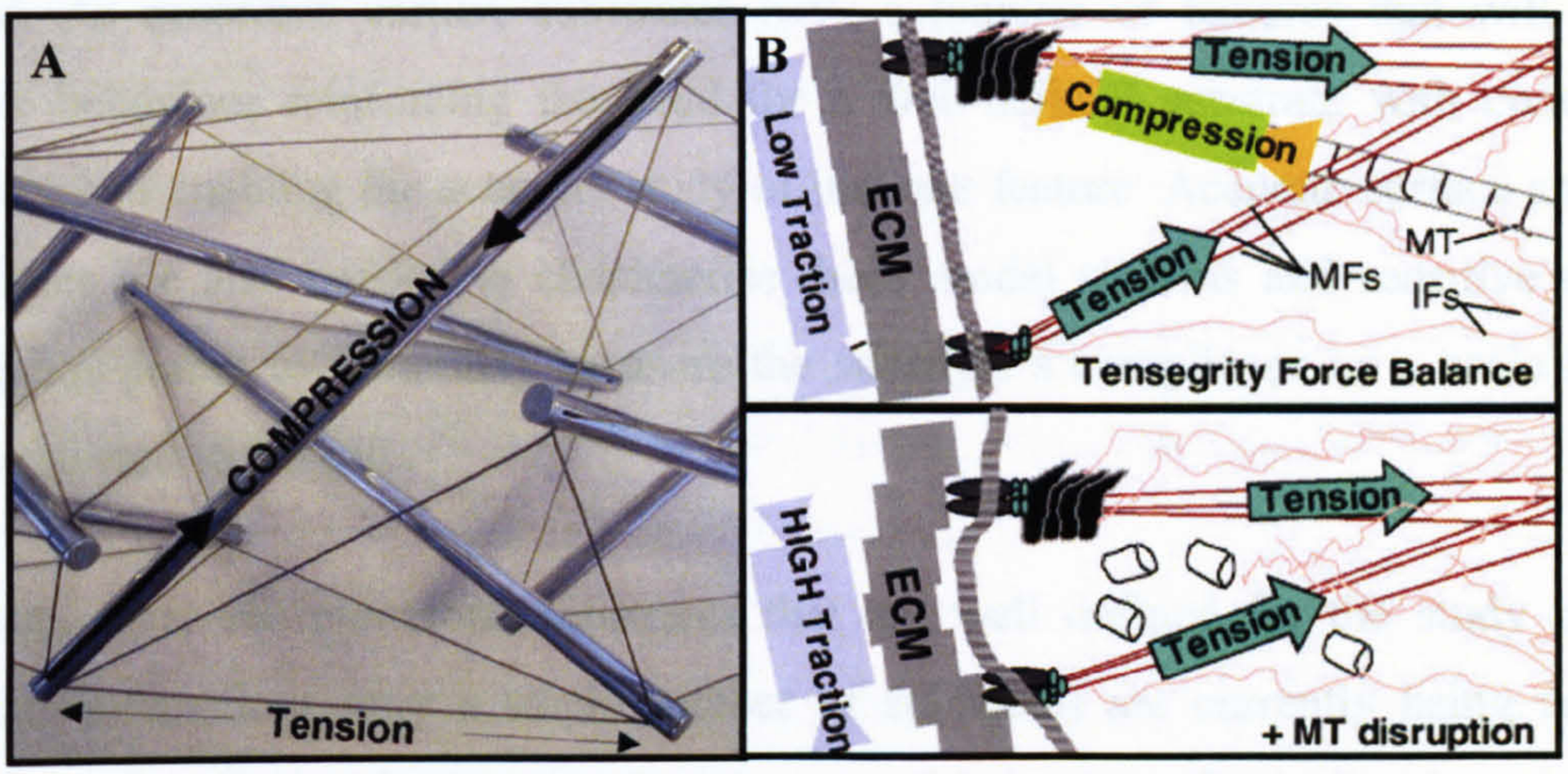


Figure 1-3: A diagram to demonstrate how the "tensegrity model" proposed by Ingber explains how physical forces applied locally to a cell are incorporated into a whole cell response. (A) shows a representative area of a Snelson sculpture with sample compression and tension elements labelled to demonstrate the tensegrity force balance based on local compression and continuous tension and (B) a representation of the same forces present in a cell's cytoskeleton (adapted from Ingber, 2003).

Ingber, (1993) has employed an architectural theory to explain how physical forces applied locally to a cell are incorporated into a whole cell response. The "tensegrity model" proposes that the whole cell is a prestressed tensegrity structure. The word tensegrity describes a pattern that results when continuous pull and discontinuous push have a balanced relationship with one another. In the cellular context it can be described that an adherent cell is in contact with the extracellular matrix and is under a "self imposed isometric tension" from the presence of a stiff actin cytoskeleton but is balanced by microtubules and external ECM adhesions (Ingber, 2003). The tensegrity theory can be used to show how an external force can be translated by a balance change in the pre-existing intracellular tension and inducing a consequent signalling event.

1.9 Substrates

To enable the study of elastic properties on cell behaviour well-defined substrates are needed. As described earlier, substrates have a number of features that will affect cellular behaviour reinforcing the need for a well-defined substrate with controlled variables and enabling the accurate study of just one feature. Accurate surface analysis techniques are also needed to characterise these model systems and sensitive tensile testing equipment to accurately measure the substrate's compliance on a scale length that is relevant to the cell.

Although there are numerous substrates that are well defined for the study of cell substrate interactions only a small number of substrates are currently being used to investigate the effects of substrate elasticity on cell behaviour. Typically, the substrates being used fall in to two categories, the natural and the artificial substrates. Natural matrices can be further divided into the proteins, for example fibrin (Bensaid et al., 2003) and collagen (Cummings et al., 2004) and the cross-linked polysaccharides for example alginate (Rowley et al., 1999) and agarose (Balgude et al., 2001). With natural substrates, the stiffness is controlled either by the amount of protein added or by the degree of cross-linking.

The artificial substrates currently being used include polyacrylamide and polydimethylsiloxane. Using an artificial system enables the study of the mechanical properties of the substrate whilst a well-defined chemistry is used. Slight differences in the surface chemistry have been shown to effect cellular attachment either directly or through differences in the adsorbed protein layer i.e. the conformation of the absorbed protein (McFarland et al., 1999), highlighting the need for tight control. The use of an artificial system eliminates any problems encountered by variance in protein concentration. Mechanical properties of artificial substrates are easily controlled by the degree of cross-linking.

Although for the purpose of this study, the materials being employed are to be used in an *in vitro* model system, their suitability as a scaffold must be considered. Disadvantages for consideration would include degradation time, cytotoxic degradation products, limited mechanical properties (Jockenhoevel et al., 2001).

1.9.1 Fibrin

As the major component of blood clots, fibrin is a biodegradable polymer that is being increasingly used in tissue engineering applications and is showing promise as an alternative scaffold in vascular tissue engineering (Jockenhoevel et al., 2001, Grassl et al., 2002).

Fibrinogen (also know as factor I) is a 340 kDa soluble plasma protein found in the blood of all vertebrates. The actions of thrombin, a serine protease converts the protein into fibrin, an insoluble gel (Walker and Nesheim, 1999).

Electron microscopic and x-ray crystallographic analysis reveals that fibrinogen is trinodular comprised of a central "E domain" and two identical outer nodules, termed the "D domains". The two D domains each consist of three non-identical pairs of disulphide bonded Aα-, Bβ- and γ- polypeptide chains, as illustrated in Figure 1-4 (Walker and Nesheim, 1999).

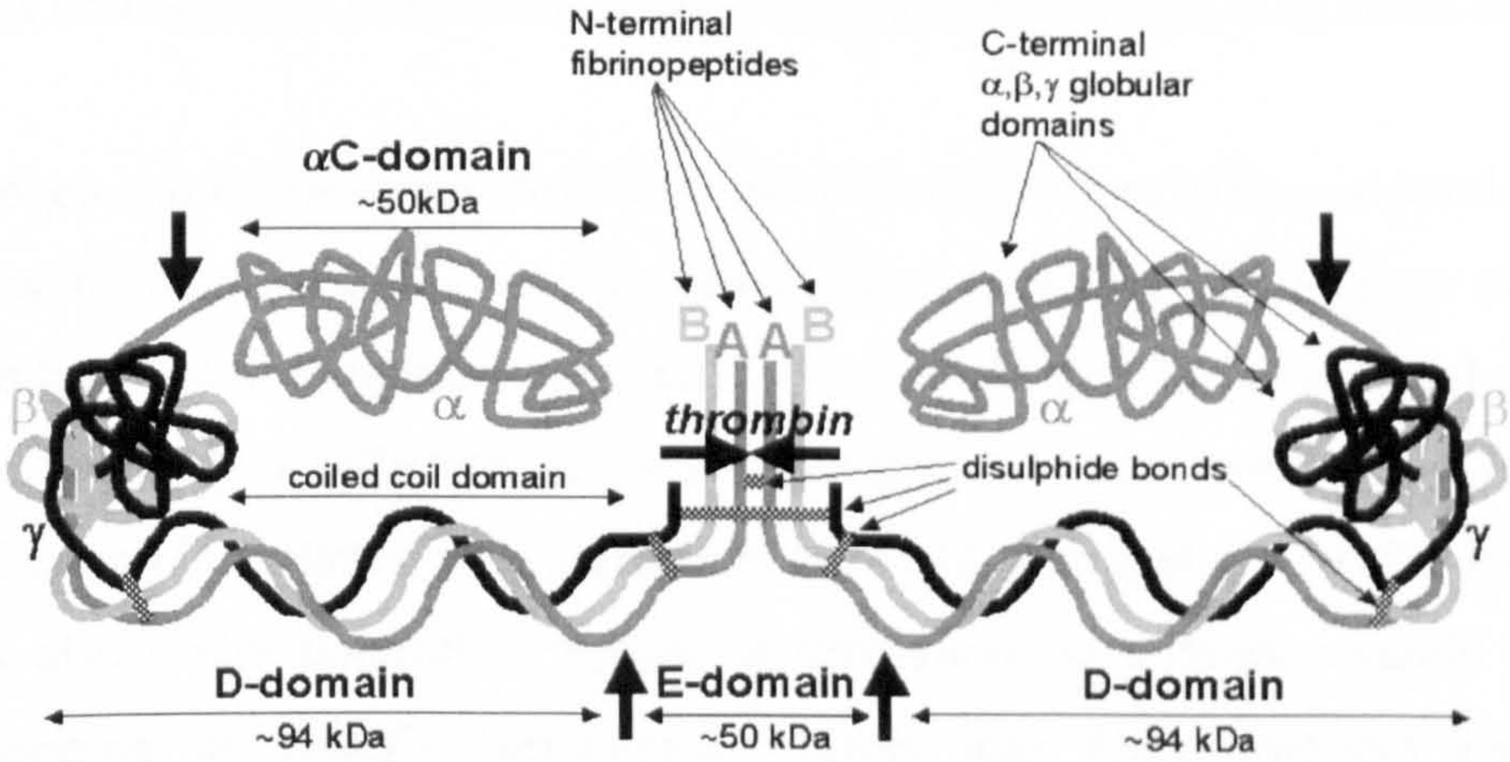


Figure 1-4: The structure of fibrinogen. The arrangement of the six chains are shown with the arrangement of the interchain disulphide bonds. Arrows indicate where plasmin acts (adapted from www.pathologyoulines.com).

The amino termini of the six chains from the D fragments join to form a small globular region in the middle of these α-helical coiled-coil chains and is so called the disulphide knot (Brown et al., 2000). This three stranded connector is where certain proteases, for

example, plasmin work yielding a small E fragmant and two larger D fragments. The amino terminals of the Aα- and Bβ- chains also include a short peptide called fibrinopeptides A and B, respectively. Fibrin is produced by the proteolytic removal of the A and B fibrinopeptides, in a sequential step, catalysed by the actions of thrombin which is produced by the activation of the proenzyme prothrombin through a series of enzymatic events in response to injury or by contact with a foreign surface.

Removal of these fibrinopeptides causes exposure of two new N-terminal sequences in the E region. These are polymerisation sites and enables another fibrinogen molecule to bind leading to the connection of fibrin monomers into two stranded, overlapping protofibrils (Walker and Nesheim, 1999). Further association of these fibrils laterally with one another forms thicker fibres eventually more and more joining together to form bundles. Strengthening then occurs when Factor XIIIa catalyses the cross-linking, by the formation of γ-glutamyl-ε-amino-lysine isopeptide bonds, between carboxyl-segments of γ-chains from D regions, within protofibrils (Brown et al., 2000). Consequently, the resulting structure or fibrin clot is composed of protofibrils, fibre bundles and fibres all arbitrarily branched which gives a highly interwoven fibrin network that acts as a haemostatic plug, acting as a trap and to stop blood loss.

Fibrinogen can be easily purified from blood and therefore offers an autologous source for a scaffold eliminating any immunological concerns (Cummings et al., 2004). Research completed suggests that mature, cross-linked fibrin, when used as a scaffold, will be relatively non-thrombogenic and unreactive to platelets thereby decreasing the risk of thrombin formation (Bordenave et al., 1992, Skarja et al., 1998). Its degradation rate is also easily controlled making it favourable as a biodegradable scaffold and incorporation of specific biochemical factors into fibrin matrices has also been demonstrated (Greisler et al., 1996). Cells are able to attach to fibrin without any further coating necessary. Research has been completed demonstrating that MSC are able to adhere, spread and proliferate when seeded into a fibrin gel with low thrombin to fibrinogen ratios (Bensaid et al., 2003). The fact that the gel is made via a quick polymerisation step would allow injection of the precursors to form the gel *in situ*.

The physical properties of the resulting fibrin clot depend greatly on the polymerisation conditions including the substrate and enzyme concentration as well as temperature, pH

and ionic strength of the polymerisation media (Oenick, 2004). The mechanical properties of fibrin have been determined via a number of different methods including, uniaxial extension (Benkherourou et al., 2000), rheometry (Ryan et al., 1999) and tensile testing (Marshall, 1978). The stiffness is typically expressed as *E* or *G* calculated by the Young's or shear moduli respectively. It remains unclear as to whether the modulus of the resultant gel is directly proportional to fibrinogen concentration. MacKintosh et al., (1995) showed that the stiffness of a polymerised network is increased as the total concentration of protein is increased and that the elastic shear modulus is approximately proportional to the square of the protein concentration.

1.9.2 Artificial flexible substrates

The use of artificial flexible substrata in cell culture is not a new idea and started over 25 years ago when Harris et al, (1980) developed a technique to use silicone rubber as a culture substratum, eliminating problems encountered by using protein substrates and their unpredictability. Although a number of artificial substrates are currently being used including polyvinyl alcohol (Cho et al., 2005) and glyoxyl agarose (Hohn et al., 1995), polyacrylamide and PDMS remain the most common due to easily controllable, physiologically relevant moduli.

1.9.2.1 Polyacrylamide

Polyacrylamide is presently the most characterised system for the study of substrate stiffness on cell behaviour. Mechanical properties are easily controlled by simply altering the monomer to cross-linker ratio with polymerisation initiated either chemically, thermally or by the addition on UV light (Wong, 2004). Other desirable characteristics including excellent optical quality permitting clear visualisation and almost ideal mechanical properties (Wang and Pelham, 1998). However, PA is chemically inert and therefore a coating must be applied to enable cell adhesion. A well-defined methodology to do this is to conjugate extracellular matrix proteins to the surface using a photoactivatable heterobifunctional reagent. Even though this method has been successfully used to support cell adhesion and to study the effects of substrate stiffness on cell behaviour (Beningo et al., 2002), it is a time consuming and expensive process. As the most characterised system, the use of PA is very attractive, however, a number of disadvantages must be considered. The range of elastic moduli attainable is limited and is not as broad a range as achievable from other substrates. Acrylamide in

the unpolymerised form is very toxic and absorbs into the skin very easily, although once the acrylamide is polymerised it is no longer absorbable. But the potential development of any model system into a scaffold must be considered. It is also important to use a range of substrates to test the hypothesis and then compare the data to make sure that it is not a surface phenomenon and that the correct conclusions are being extrapolated from the results.

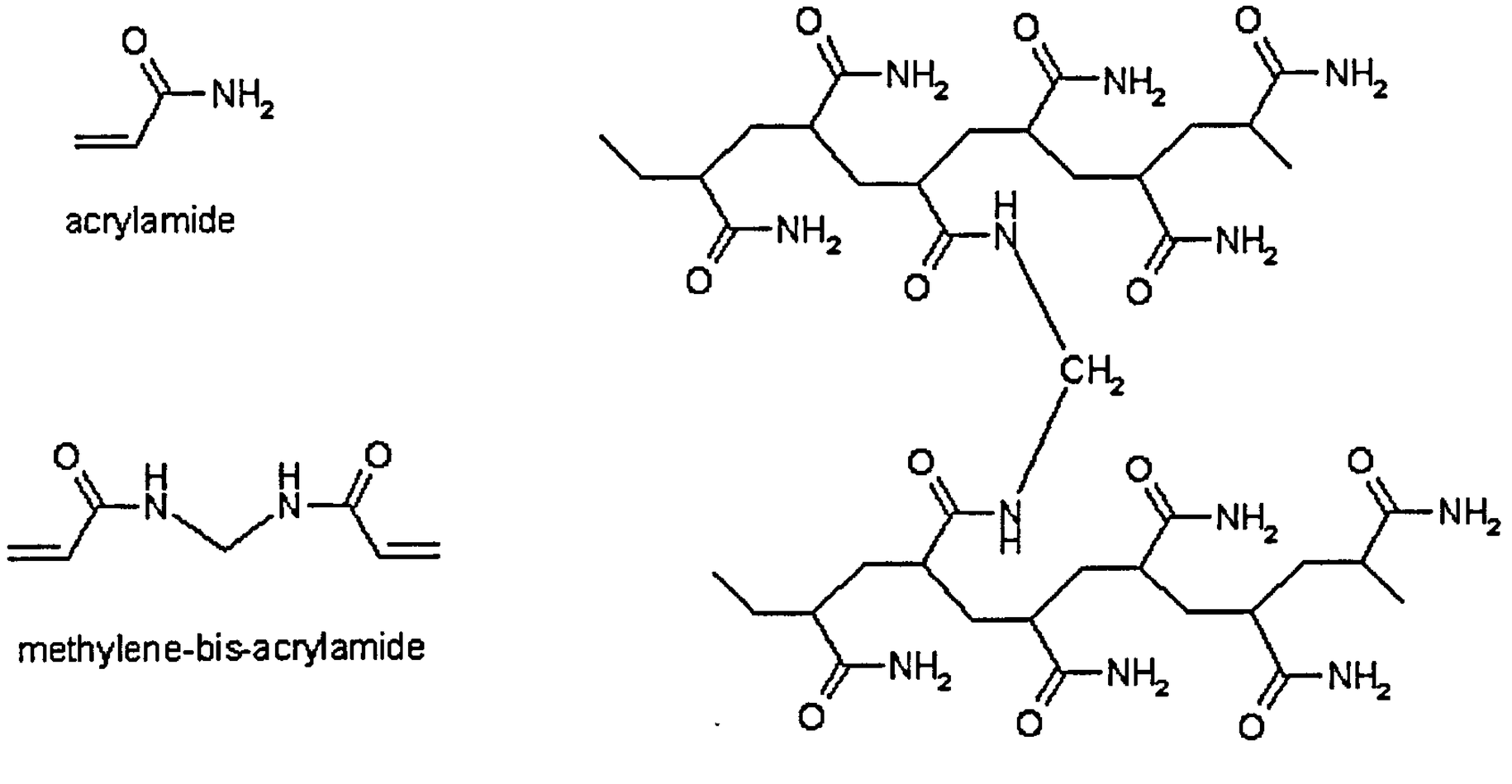


Figure 1-5: The formation of polyacrylamide. Polyacrylamide is made by crosslinking acrylamide typically with bis-acrylamide. Polymerisation can be initiated chemically, thermally or by a photoinitiator (adapted from http://www.steve.gb.com/).

1.9.2.2 Silicone

Silicone is already used extensively as a biomaterial in a number of applications including contact lenses (Hill and Schoessler, 1967), catheters (Haid et al., 1988), heart valves (Mullison, 1964), artificial limbs (Marks and Michael, 2001) and breast implants (Weiner et al., 1974). Its good optical clarity, transparency, low permeability to water and its thermal stability contribute to making it a highly desirable biomaterial (Lee et al., 2004). These properties along with the large range of Young's moduli that can easily be created have led to its use in the study of elastic substrates on cell behaviour.

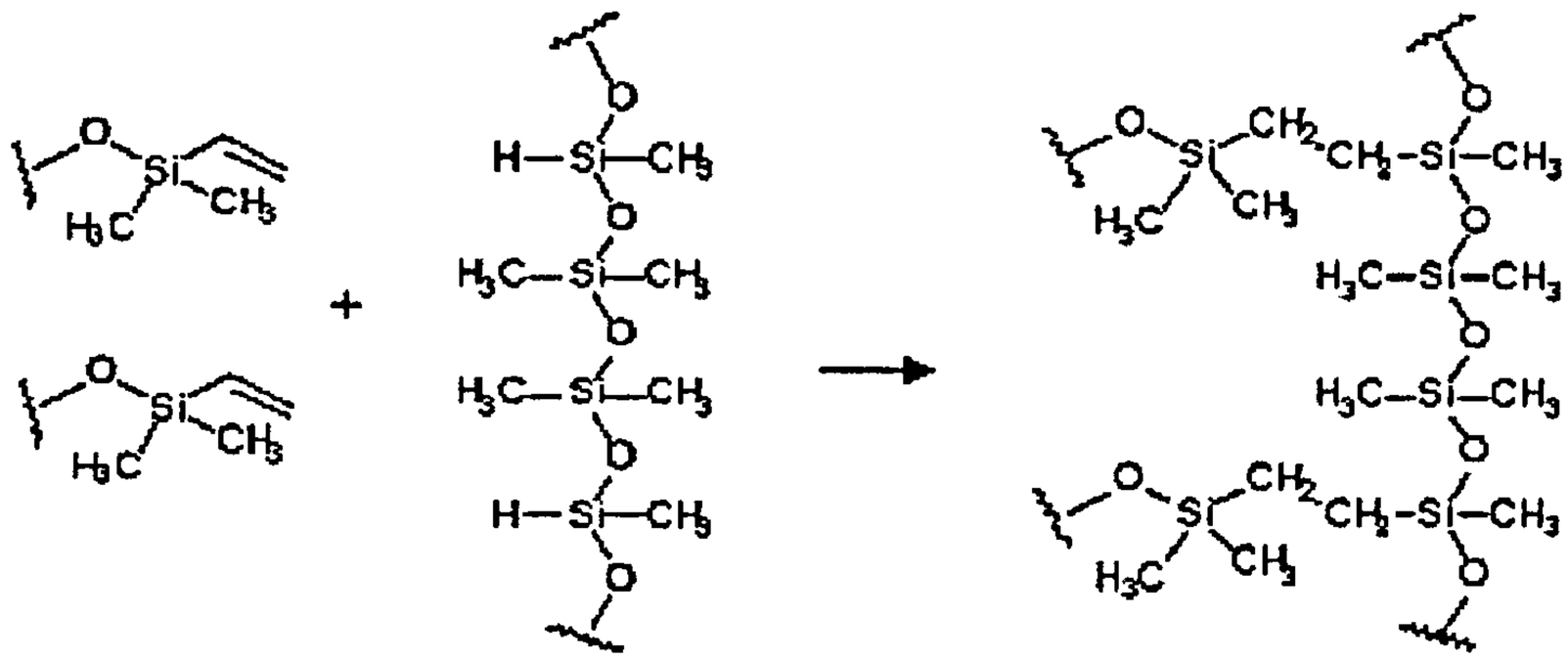


Figure 1-6: The formation of PDMS. PDMS is created by crosslinking siloxane oligomers. Varying the ratio of siloxane oligomer monomer to siloxane crosslinker alters the mechanical properties of the resultant gel by changing the degree of crosslinking (adapted from Wong, 2004).

Silicone rubber films can be cast from polydimethylsiloxane with the siloxane oligomers and siloxane cross-linkers varied to obtain a variety of mechanical properties. The mechanical properties of PDMS can easily be controlled by simply altering the ratio of siloxane oligomers and cross-linkers, creating a large range of Young's moduli. With possible moduli of greater than 1 MPa, PDMS offers a more physiological range than the other substrates and is 10x stiffer than the largest moduli obtainable from polyacrylamide. Like polyacrylamide, PDMS is cast and used as a thick film however polyacrylamide is used attached to glass where PDMS can be cast and used independent of the glass.

Even though there are reports of cells adhering to an unmodified silicone substrata (Harris et al., 1980), cell adhesion is poor and has been attributed to the hydrophobicity (due to closely packed side methyl chains), chemical inertness and high chain mobility (Wong, 2004). Most other investigators have relied on non-covalent methods to enhance cell adhesion *i.e.* physisorption of fibronectin (Gray et al., 2003), however, recent studies found adsorption of matrix proteins to PDMS to be inefficient to enhance cell

attachment (Cunningham et al., 2002). Other methods to enhance cell adhesion are summarised below.

The most fundamental approach (as applied to tissue culture grade polystyrene) is oxygen treatment, creating OH at the surface, to render the surface hydrophilic. However, using this method for the treatment of PDMS tends to be problematic with reversion in air.

Migonney et al., (1995), enhanced cell attachment onto silicone by using chemical immobilisation. Epoxy ring-opening functionalisation of the silicone at random sites along the chain was demonstrated to support fibroblast attachment and growth. Reports of the system being unstable have been made and would therefore not permit the long-term culture of cells.

Gelatin is widely used in the enhancement of cell attachment. Crosslinking gelatin can be achieved by either enzymatically or chemically and renders the gelatin harder and more stable at higher temperatures, a technique commonly employed in the biomaterials field. Ai et al., (2002) applied the technique, of glutaraldehyde crosslinked gelatin, to silicone rubber creating a hydrophilic film and improved endothelial attachment and growth. Although the technique was successful to enhance cell adhesion, it was reportedly 135 μm thick and would possible not allow the cells to feel the mechanical properties of the underlying material.

Lee et al., (1996) employed plasma-induced grafted polymerisation to introduce polyacrylic acid onto argon-plasma treatment silicone rubber membranes. Reducing the contact angle of the typically hydrophobic material to approximately 45-50 degrees. The attachment of cells onto polyacrylic acid -grafted surfaces was clearly observed and peusopoda occurred.

More recently, Ai et al., (2003) have devised a method to coat a nanometre-thick film on to the silicone by varying adsorption of oppositely charged components. This layer-by-layer treatment uses alternating layers of the polycation polyethyleneimine and the polyanion polystyrene sulphate and has been shown to support cell adhesion for up to

two weeks. Although the technique allows the coating of substrates any size or shape, the technique is labour intensive and time consuming.

1.10 Plasma polymerisation

Plasma polymerisation is a gaseous phase technique employed to coat pinhole free, nano-thin polymeric films (typically 10-100 nm) on to surfaces to enhance cell adhesion without compromising the bulk characteristics of the material. The technique is very attractive in the field of biomaterials as the plasma deposition is possible onto almost any solid material, with little or no pre-treatment and exact control over coating thickness is possible (Daw et al., 1998). Plasma treatment is also employed for coating blood-compatible surfaces for example in vascular grafts, stents and heart valves, for non-fouling surfaces such as contact lenses and biosensors and for barrier coatings used for drug-release or corrosion protection (Chu et al., 2002). Once the plasma polymerisation apparatus is set up and optimised; deposition is quick and relatively simple yielding a clean, sterile surface for subsequent cell culture.

Plasma polymerisation is the transformation of low-molecular weight molecules ('monomer') into high-molecular weight molecules ('polymer'). The technique requires the supply of power, for example a radio frequency or microwave signal onto the desired substrate. This power is then matched in both phase and amplitude to the corresponding potentials in the plasma. The plasma polymer deposited is comprised of complex units from the initial monomer, which are often fragmented, cross-linked and rearranged and unlike the original repeating monomer unit (Chu et al., 2002). The amount of monomer fragmentation, and consequently surface composition, can be controlled either by changing the monomer flow rate or the input power. It is therefore possible to modify the deposition to create the desired surface (Detomaso et al., 2005).

A number of monomers have been shown to enhance cell adhesion. Previous work has shown the importance of negatively charged groups especially carboxylic acid in promoting cell adhesion (Maroudas, 1975). Acrylic acid is the simplest unsaturated carboxylic acid and plasma deposited acrylic acid coatings have previously been used in cell culture experiments and have been shown to support the adhesion and growth of a number of cell types including 3T3 fibroblasts (Detomaso et al., 2005), ROS 17/2.8 osteoblast-like cells (Daw et al., 1998) and human keratinocytes (France et al., 1998).

As previously described, surface chemistry of the substratum is very important and has been shown to affect a number of cellular processes. The interaction between the

biomaterial and protein is paramount. Proteins present in the serum adsorb rapidly to the material and it is the surface chemistry present that dictates the composition of the layer formed and the conformation of the proteins within it. Hence, subsequent cellular functions are dependant upon it (Schakenraad et al., 1986). Another important consideration is the stability of the coating over time in culture and a critical issue for stem cells that they need a coating that is stable in culture for extended periods.

1.11 Methods to characterise mechanical properties of substratum

Many different methods are currently used to measure the mechanical properties of the different cell culture substratum. Regardless of the method, the resistance of a solid to an applied stress is measured as the solids elastic modulus (E) (Discher et al., 2005). The most basic method used to calculate E is obtained by simply applying a force (typically a weight of known mass) to the material and then measuring the materials change in length (Wang and Pelham, 1998). More refined techniques include indenting the material using macro-indenters or microindentation (Pelham and Wang, 1997) including atomic force microscopy (Engler et al., 2004b). Rheometry (Flanagan et al., 2002) and uniaxial tension (Gray et al., 2003) have also been employed to determine either the elastic properties with most researchers determining the Young's or shear modulus. Consideration must be given when choosing the technique as to the most reliable measurements obtainable. Bulk properties measured by uniaxial tension, for example, will measure the bulk elastic properties and therefore not allow for heterogeneities in the substrates in the micron scale. More localised elastic properties measured on the micron scale will give a more accurate measurement and allow greater consideration for the moduli in the cells local microenvironment. Table 1-1 summarises the methods and techniques previously used to calculate the mechanical properties for both PDMS and fibrin substrates.

Although the influence of fibrinogen concentration on the mechanical properties of the resultant gel has been previously studied the data presented is often conflicting, which is probably due to ambiguous and diverse methodologies. The mechanical testing data for PDMS, although not conflicting, is often measured as a bulk measurement and therefore does not allow for the heterogeneitic nature of the polymer on a more localised scale. The data in Table 1-1 highlights the need for a more controlled method of calculation to

allow comparisons between different research groups and therefore the true extrapolation of results.

Table 1-2 shows the broad range of elastic moduli found throughout the adult organism and highlights not only the need for the production of physiologically relevant moduli but again the need for a consensus on the technique and method of calculating the mechanical properties of tissue enabling comparison of data and the progression of research in the field.

1.12 Endnote

Understanding the effects that the stiffness of the underlying substratum have on MSC will have important implications not only within tissue repair strategies for tissue engineering purposes but also in understanding the basic biology of the cell. The aim of the project was to develop and characterise variable-stiffness substratums and thereby create more physiological growth conditions for MSC in culture. Use of the culture systems enabled the effects of substrate stiffness on MSC to be investigated, to test the hypothesis that "substrate stiffness will effect MSC proliferation and differentiation".

	Substrate	Modulus	Method	Reference
Fibrinogen Concentration (mg/ml)	0.5-3	0.93-6.49 kPa	Uniaxial extension	(Benkherourou et al., 2000)
	0-6	0-0.15 kPa	Rheometer	(Ryan et al., 1999)
	30-70	6.7-23.9 kPa	Uniaxal extension	(Sierra et al., 2002)
	25-75	2-fold increase	Tensile testing	(Marshall, 1978)
	16-28	12 kPa	Tensile testing	(Velada et al., 2002)
PDMS (Crosslinker : base)	1:50-1:10	12 kPa-2.5 MPa	Uniaxial tension (5- 30 % strain)	(Gray et al., 2003)
	1:50-1:10	48 kPa –1.78 MPa	Tensile testing (0.1-0.5 % strain)	(Brown et al., 2005)
	1:50-1:10	12 kPa- 1 MPa	Tensile testing (70 % elongation)	(Balaban et al., 2001)

Table 1-1: A table to summarise published data of Young's moduli for different PDMS and fibrin substrates and the method used.

Tissue	Modulus (kPa)	Method	Model	Reference
Extensor digitorum longus	12 ± 4	AFM	mouse	(Engler et al., 2004b)
Myocytes (C2C12 cells)	12-15	AFM	mammalian	(Collinsworth et al., 2002)
Fibroblasts	2-5	AFM	human	(Bushell et al., 1999)
Saphenous vein	27	NS	human	(Wong, 2004)
Artery: Thoracic aorta Abdominal aorta Carotid a.	 1000 1000 1000-3000	NS	human	(Wong, 2004)
Bone tissue	~18	NS	human	(Liu, 2005)
Brain tissue	~2.5	NS	human	(Liu, 2005)
Grey matter	0.2	shear	human	(Flanagan et al., 2002)
Articular cartilage	100-1000	compressive	bovine	(Bonassar et al., 1995)

Table 1-2: Elastic moduli of various tissues from a variety of species. NS= not stated.

CHAPTER TWO: Materials and Methods

2.1 Cell culture

All chemicals were purchased from Sigma-Aldrich (Dorset, UK), unless otherwise stated and used to the manufactures instructions.

2.1.1 Cell culture medium preparation

Working strength DMEM (Cambrex Bio Science, Workingham, UK) was supplemented with 10 % Serum supreme (Cambrex Bio Science, Workingham, UK), 1 % Ultra-glutamine (Cambrex Bio Science, Workingham, UK), and 1 % penicillin-streptomycin solution (final concentrations: 100 units/ml and 100 μg/ml, respectively), and will hereafter be referred to as growth medium. For osteogenic differentiation cells were cultured in growth medium supplemented with dexamethasone at final concentration 10^{-8} M and ascorbic acid 50 μg/ml.

2.1.2 Isolation of rat bone marrow stromal cells

All animal procedures were carried out according to current Home Office regulations and all necessary licenses had been obtained. Male Wistar rats (Harlan, UK Limited) (125- 200 g, 8 weeks old) were killed by cervical dislocation. Male rats are typically used in order to minimise changes in hormone levels seen during the estrous cycle of female rats. Tibiae and femurae were removed under aseptic conditions and all soft tissue removed. Bone marrow cells were then recovered from the bone by centrifugation according to the method of Dobson et al., (1999b). This method was chosen as more of the bone marrow is recovered as compared to the standard flush method (**Figure 2-1**) (Dobson et al., 1999b). The isolated bones were placed within small plastic supports in microfuge tubes and centrifuged at 2000 rpm for 2 minutes (**Figure 2-2**). The marrow cells pelleted at the bottom of the tube were then resuspended in 0.5 ml growth medium, pooled and the volume made up to 10 ml growth medium per bone.

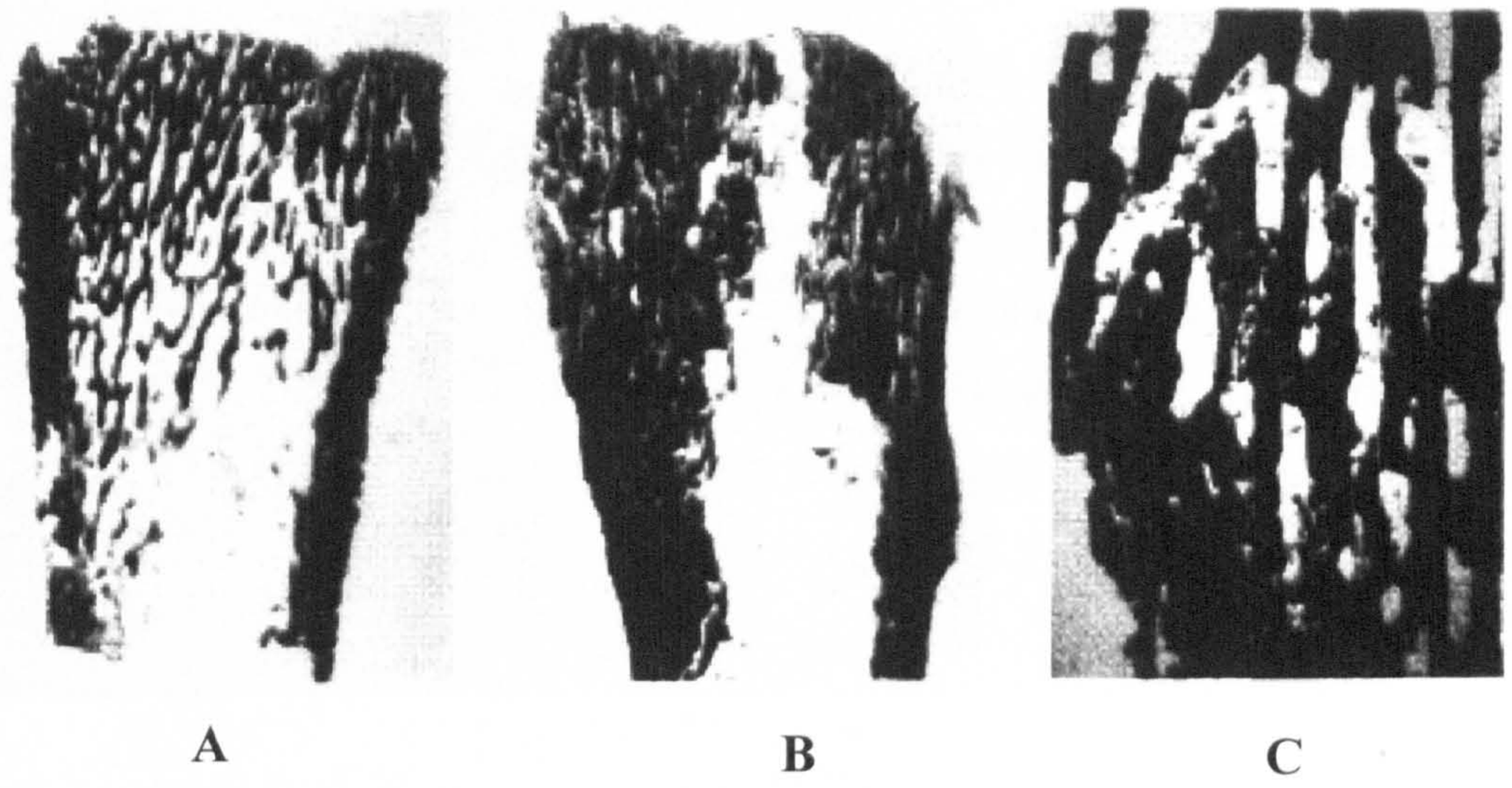


Figure 2-1: Histological examination of tibiae after different bone marrow extraction methods. Centrifugal method of bone marrow extraction A) removes more bone marrow compared to the standard flushing method B). Higher magnification reveals that only bone lining cells remain in the centrifuged bone C). (Image used with permission from Dobson et al., (1999b).

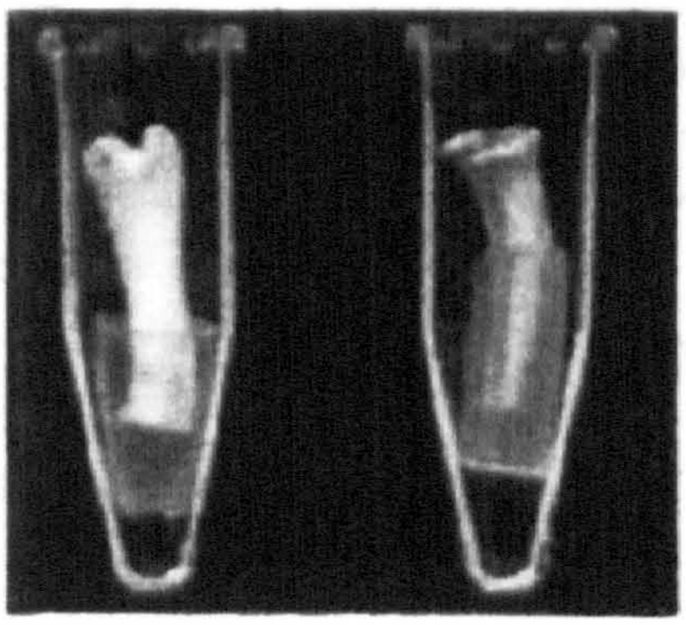


Figure 2-2: Recovery of bone marrow cells from rat tibiae and femurae using centrifugation. Excised tibiae and femurae were place in microfuge tubes, supported by plastic inserts and centrifuged at 2000 rpm for 2 minutes. The bone marrow pellets was resuspended in 1 ml growth medium and pooled. (Image used with permission from Dobson et al., (1999b).

2.1.3 Fibroblastic colony forming unit assay (CFU-f)

Principle: The CFU-f assay has become the standard assay for *in vitro* analysis and determination of the potential differentiation to cells from mesenchymal tissues. Cells obtained from the bone marrow are plated at very low densities, the adherent population is expanded and colonies resulting from the single cells are then scored. A presumption is made that each colony is derived from a single cell precursor. The differentiation potential can then be ascertained by induction, specific staining and subsequent quantification of the positive colonies (**Figure 2-3**).

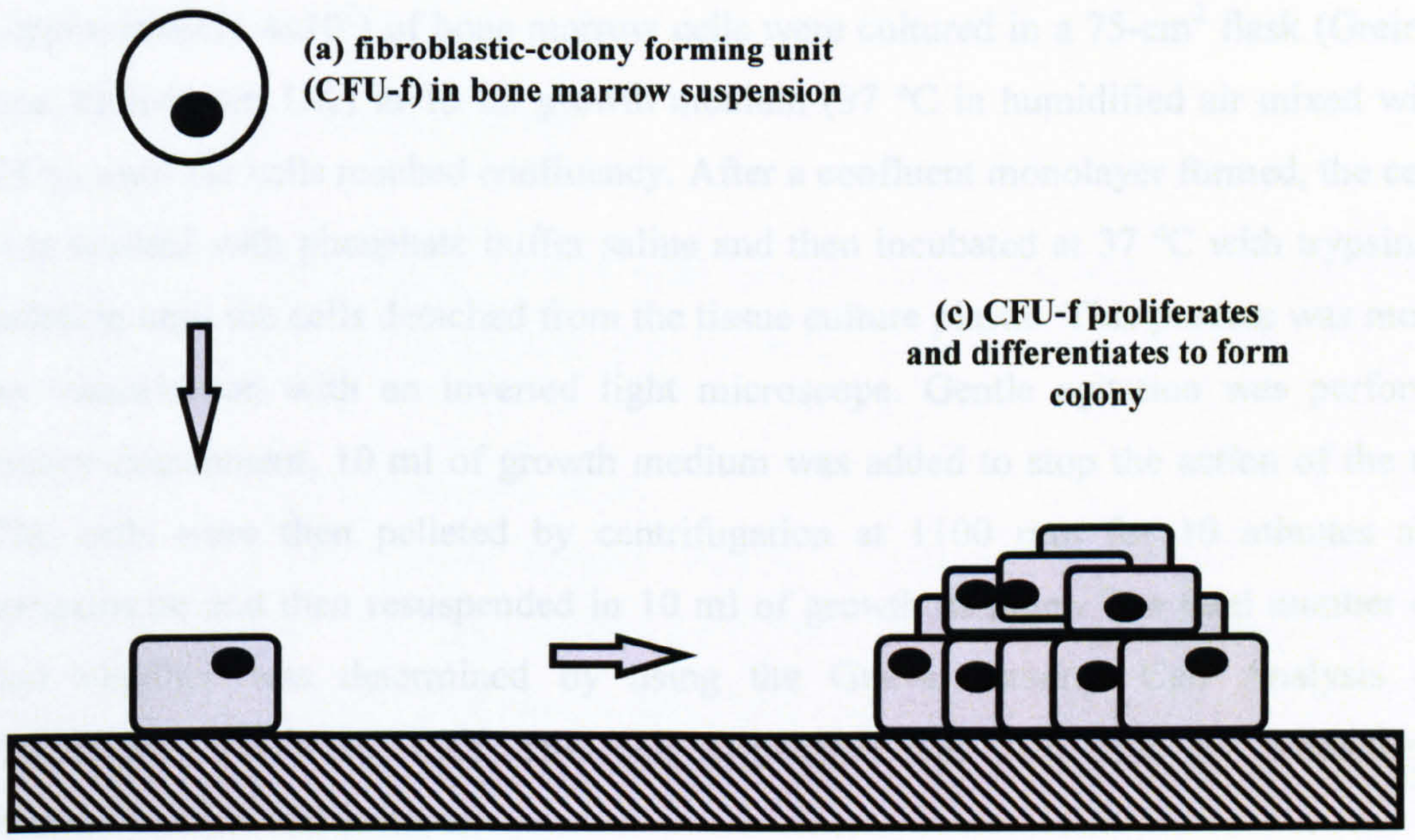


Figure 2-3: A schematic diagram of the CFU-f assay used to determine the differentiation potential of mesenchymal tissue. Bone marrow cells are added to a 55-cm^2 culture dish, the CFU-f's adhere and proliferate to form a colony. Subsequent staining allows for the identification of colonies.

Procedure: CFU-f cultures were performed as described previously (Dobson et al., 1999a). 500 µl of freshly isolated bone marrow cells (approximately 2 x 10^6 cells) were plated out in 55-cm^2 tissue culture dishes (Greiner bio-one, Gloucester, UK) containing the required culture substrate to be tested. 10 ml of osteogenic growth medium was added and the plate incubated (37 °C, 7 % CO_2) for five days at which point the cell culture medium was

changed. Cell culture medium was changed twice weekly and the cultures maintained for 14 days after which cells were washed with PBS, fixed by the addition of 70 % IMS (BDH, Dorset, UK) and subsequently stained.

2.1.4 Secondary Bone Marrow Stromal Cell Culture

Secondary (2°) bone marrow cell cultures are used to provide a more homogeneous cell population enriched for MSC (Scutt and Bertram, 1995). Blood cells are typically not adherent and are therefore washed away at the first medium change. Any adherent monocytes are lost during the first passage. 10 ml of the single cell suspension (approximately $4x10^7$) of bone marrow cells were cultured in a 75-cm^2 flask (Greiner bio-one, Gloucester, UK) in 15 ml growth medium (37 °C in humidified air mixed with 7 % CO_2), until the cells reached confluency. After a confluent monolayer formed, the cell layer was washed with phosphate buffer saline and then incubated at 37 °C with trypsin/EDTA solution until the cells detached from the tissue culture plastic. This process was monitored by visualisation with an inverted light microscope. Gentle agitation was performed to ensure detachment. 10 ml of growth medium was added to stop the action of the trypsin. The cells were then pelleted by centrifugation at 1100 rpm for 10 minutes at room temperature and then resuspended in 10 ml of growth medium. The total number of cells and viability was determined by using the Guava Personal Cell Analysis (Guava Instruments, Hayward, CA). This cell suspension was then used for secondary bone marrow stromal cell cultures.

2.1.4.1 High-density monolayer cultures

Principle: High-density cultures are used in contrast to the CFU-f, where cells are seeded at very low plating densities. In high-density cultures cells proliferate and interact forming cell-to-cell contacts.

Procedure: Secondary bone marrow cells were plated out into 2-cm^2 wells (Greiner Bio-one, Gloucester, UK) at a density of 2×10^4 per well in 500 µl of osteogenic growth medium and incubated at 37 °C (7.5 % CO_2). Medium was changed on day 5 and every

three days, thereafter. Cultures were stopped on day 10 by fixation and subsequently analysed as described below.

To study of the effects of certain inhibitors of cellular function on the differentiation of MSC the following reagents were added to 2° MSC seeded at a density of 2 x 10^4 after an initial 24-hours in culture; mitomycin (50 μg/ml), cytochalasin (1 μg/ml) and apidicolin (2 μg/ml). Y27632 (10 μM), blebbistatin (50 μM) and SB203580 (10 μM) were added after an initial 24-hour culture period to cells seeded at a density of 1 x 10^4. After a further 7 days, cells were fixed and stained for ALP expression (as described below).

2.2 Histochemical staining

2.2.1 Fixation

Cultures were terminated by washing with PBS and then by the addition of IMS (70 %) for 5 minutes. The cultures were rinsed with tap water before staining.

2.2.2 Alkaline phosphatase staining

Principle: The non-specific enzyme alkaline phosphatase hydrolyse Naphthol ASBI phosphate disodium salt under alkaline conditions. The reaction product naphthol can then be coupled to Fast red or Fast blue, a diazotized compound to produce a coloured, insoluble end product that can then be visualised to determine the number of alkaline phosphatase positive colonies within the culture.

Procedure: Alkaline phosphatase staining solution: 100 mg Naphthol phosphate ASBI was dissolved in 180 ml Tris buffer (20 mM), 10 ml dimethyl formamide was added and then the solution was made up to 500 ml with distilled H_2O and buffered to pH 8.5 using NaOH (1 M). Just before use, Fast red (1 mg/ml) was added. 5 ml was added to each previously fixed culture dish and gently shaken (30 rpm) at room temperature on an orbital shaker for 30 minutes. After staining, dishes were rinsed with tap water before allowing to air dry. The plates were subsequently photographed to document the amount of ALP staining. To allow for further staining the plates were destained by gently shaking with 100 % IMS overnight on the orbital shaker (30 rpm).

2.2.3 Total colony staining

Principle: Methylene blue binds stoichiometrically to basic histone proteins and therefore all cells within the individual colonies are stained. Total colony number can then be determined by counting the colonies.

Procedure: Previously fixed plated were rinsed with borate buffer (10 mM boric acid; adjusted to pH 8.8 with NaOH). 5 ml of total colony staining solution (1 mg/ml methylene blue in 10 mM borate buffer, pH 8.8) was added to each plate and gently shaken (30 rpm) at room temperature for 30 minutes. After staining the dishes were rewashed three times with borate buffer. Plates were left to air dry and then photographed for future analysis of total colony number.

2.2.4 Image requisition and analysis

After staining, the dishes were photographed over a white light transilluminator using a digital camera. The acquired digital images were imported into "Photoshop", colony irregularities smoothed out and converted into a 256 level greyscale format. The image was then analysed using Bioimage "Intelligent Quantifier" image analysis software which automatically locates and quantifies each individual colony according to size and intensity (Dobson et al., 1999a).

2.3 Analysis of high-density cultures

2.3.1 Quantitative measurements of ALP

Principle: The enzyme ALP will hydrolyse the substrate para-nitrophenol phosphate (p-NPP) to para-nitrophenol (p-NP) under alkaline conditions rendering a yellow complex with which the optical density is linearly proportional to the ALP activity.

Procedure: Fixed cells from secondary BMSC cultures were incubated with 750 µl of ALP assay buffer (5 mM ρ-NPP and 50 mM tris/HCl, pH 9.5). After incubating at room temperature for 5 minutes, 100 µl of the supernatant was transferred to 96-well plates (0.31 cm^2) (Greiner Bio-one, Gloucester, UK). Absorbance was measured at 405 nm using a micro-plate reader. Assay buffer was used to determine background readings.

The cells were destained with 100 % IMS for 5 minutes, rinsed with PBS and cell number subsequently determined using the methylene blue assay as described below.

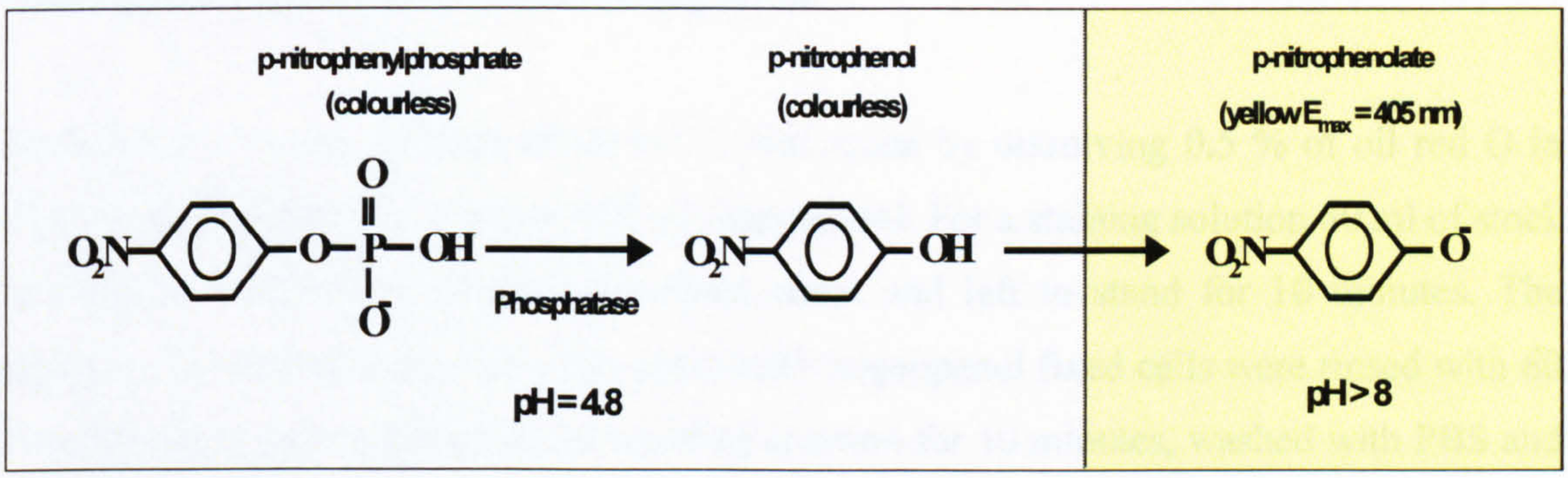


Figure 2-4: The enzyme ALP hydrolyses para-nitrophenol phosphate to para-nitrophenol under alkaline conditions. The resultant yellow solution can then be measured spectroscopically at an optical density of 405 nm to determine ALP activity.

2.3.2 Methylene blue cell proliferation assay

Procedure: Methylene blue binds stoichiometrically to basic histone proteins and therefore stains all cells present blue. The optical density of the eluted stain is proportional to cell number.

Principle: To analyse cell number, methylene blue staining was performed. Cells in each well were stained at room temperature for 30 minutes with 500 µl of 0.1% (w/v) methylene blue. Any unbound dye was rinsed off with an excess of distilled water. After air drying, the bound stain in each well was extracted at 37 °C for 30 minutes with acid ethanol (1% HCl in IMS) 300 µl. The solution was then transferred to a 96-well (0.31 cm^2) plate and the absorbance was measured at 630 nm using a micro plate reader. The background reading was determined from culture samples containing no cells, but treated using the same procedures.

2.3.3 Oil red-O staining

Principle: Differentiation of MSC to adipocytes is typically observed by the presence of lipid filled vesicles. Oil red-O (1-8-[4-(Dimethylphenylazo)dimethylphenylazo]-2-naphtalenol) is a weakly acidic, diazo oil-soluble dye that stains neutral fats and is therefore used for histological identification of adipocytes.

Procedure: A stock solution of oil red O was made by dissolving 0.5 % of oil red O in absolute isopropanol. i.e. 0.5 g in 100 ml isopropanol. For a staining solution 60 ml of stock solution was diluted in 40 ml of distilled water and left to stand for 10 minutes. The solution was filtered before use. The previously isopropanol fixed cells were rinsed with 60 % isopropanol and immersed in the staining solution for 10 minutes, washed with PBS and allowed to dry for subsequent analysis.

2.4 Quantification of cell attachment and visualisation

2.4.1 Cell attachment

Principal: The measurement of cell attachment is commonly used to study the interaction of cells with biomaterials and to assess their suitability as a cell culture substratum. MSC cell division typically occurs after 24 hours, so cells were counted before this time-point to ensure the accurate quantification of cell attachment.

Procedure: Cells were seeded on to the different substrates at a density of $2x10^4$ and incubated at 37 °C (7.5 % CO_2), at 3 and 24 hour time-points the substrates were washed with PBS fixed in cold ethanol and rewashed three times in PBS to ensure all unattached cells were removed. Cells were then counted by means of one of two methods.

1) Cells were fixed, washed and stained with methylene blue. The numbers of attached cells were determined by direct counting under an inverted light microscope in phase-contrast. 5 fields of view were counted per well and 6 wells were counted for each sample.

2) Alternatively, cells were fixed, washed and stained with DAPI (15 μm) for 10 mins rewashed before automated counting was preformed using an Imagexpress 5000A (Molecular devices, CA, USA) and Imagexpress Console 1.0 software.

2.4.2 F-actin staining

Principal: Phalloidin, a cell toxin, acts by binding actin filaments together and thereby preventing its depolymeriastion. This property makes phalloidin a useful tool in the visualisation of the actin cytoskeleton. Phalloidin tagged with a fluroscent analog are commonly used for the visualisation of the actin filaments using immunoflurescent microscopy.

Procedure: Cells, which had been previously fixed, were washed with PBS before the addition of 300µl 0.1 % triton X for 20 minutes at room temperature. After three further rinses, 300 µl phalloidin (3 µM) diluted in PBS was added and incubated for 30 minutes at room temperature with the exclusion of light. After two more rinses the cells were incubated with 300 µl DAPI (15 µm) for 10 minutes. The cells were finally rinsed once more with PBS before visualisation using an Imagexpress 5000A (Molecular devices, CA, USA), and Imagexpress Console 1.0 software.

2.5 Proliferation studies

2.5.1 Fluorescence-activated cell sorter analysis- growth curve and cell viability

Principle: Guava ViaCount reagent, a commercially available product, was used to determine total cell counts and the percentage viability of the cells within the population. The Guava ViaCount assay distinguishes between viable and non-viable cells by incorporating a DNA- binding dye with the staining being determined by the differential permeability of the cell membrane. A first fluorochrome enters all cells and binds to the DNA a second fluorochrome, however, can only enter dead, permeable cells. Each fluorochrome is detected at a different wavelength with flow cytometry used to determine the percentage of dead cells within the total population.

Procedure: The growth curve was performed on primary cells cultured on fibrin or TCP for 11 days. 250 µl of a suspension of freshly isolated whole primary bone marrow cells was seeded on to fibrin coated 24 well plates or TCP in growth medium. This amount was used as it was known to give rise to a confluent monolayer after 11 days. To calculate cell number and viability cells were washed three times with PBS, detached with EDTA/trypsin

and re-suspended in 5 ml filtered medium (10 % serum). After a 5 minute incubation (at 37 °C) with the via-count solution (1:10 dilution) (Guava Technologies, Hayward, CA), cell number and the percentage of viable cells was determined in a Guava flow cytometer (Guava Technologies, Hayward, CA),

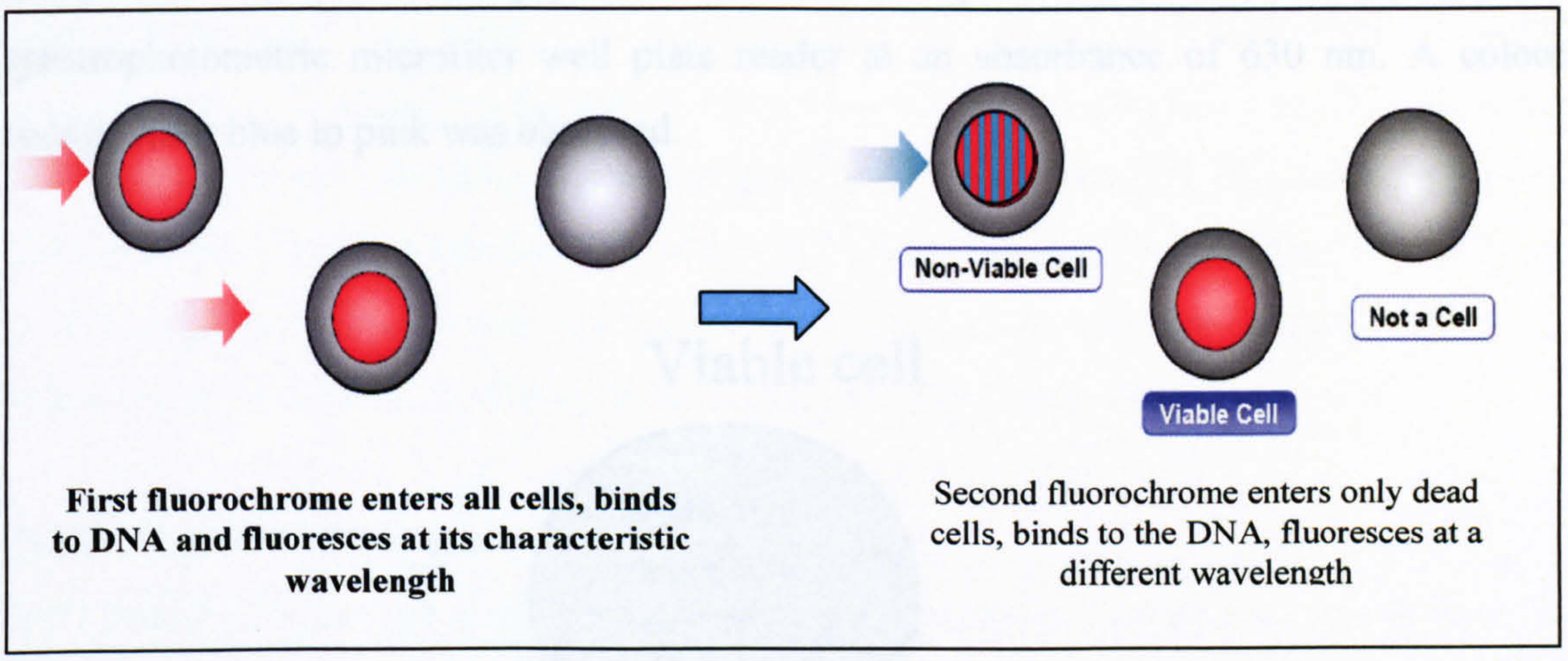


Figure 2-5: Guava Viacount reagent works on the principal that the permeability of the cell membrane is different in live and dead cells. A first fluorochrome enters all cells, binding to the DNA. A second fluorochrome only enters dead, permeable cells and fluoresces at a different wavelength. Flow cytometry is used to determine the percentage of dead cells (adapted from www.guavatechnologies.com).

2.5.2 Alamar Blue™ Assay

Principle: Alamar Blue™ is used to measure quantitatively the proliferation of cells. It works by incorporating a colourimetric growth indicator based on detection of metabolic activity. AlamarBlue™ works on the principle that the internal environment of the proliferating cell is more reduced than that of non-proliferating cells with the ratios of NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD all increasing as the cell proliferates. AlamarBlue™, diluted within the cell culture medium, is reduced by these metabolic intermediates which is accompanied by a shift in colour from the oxidized blue to the reduced pink state (**Figure 2-6**).

Procedure: AlamarBlue™ (CambrexBio Science, Workingham, UK) was added, to the each well of a 24-well plate, to an end concentration of 10 % of the total volume. Cultures were returned to the incubator for 6 hours at which point 100 µl of the medium and alamarBlue™ solution was pipetted into a 96-well plate (0.31 cm^2) and read in the spectrophotometric microtiter well plate reader at an absorbance of 630 nm. A colour change from blue to pink was observed.

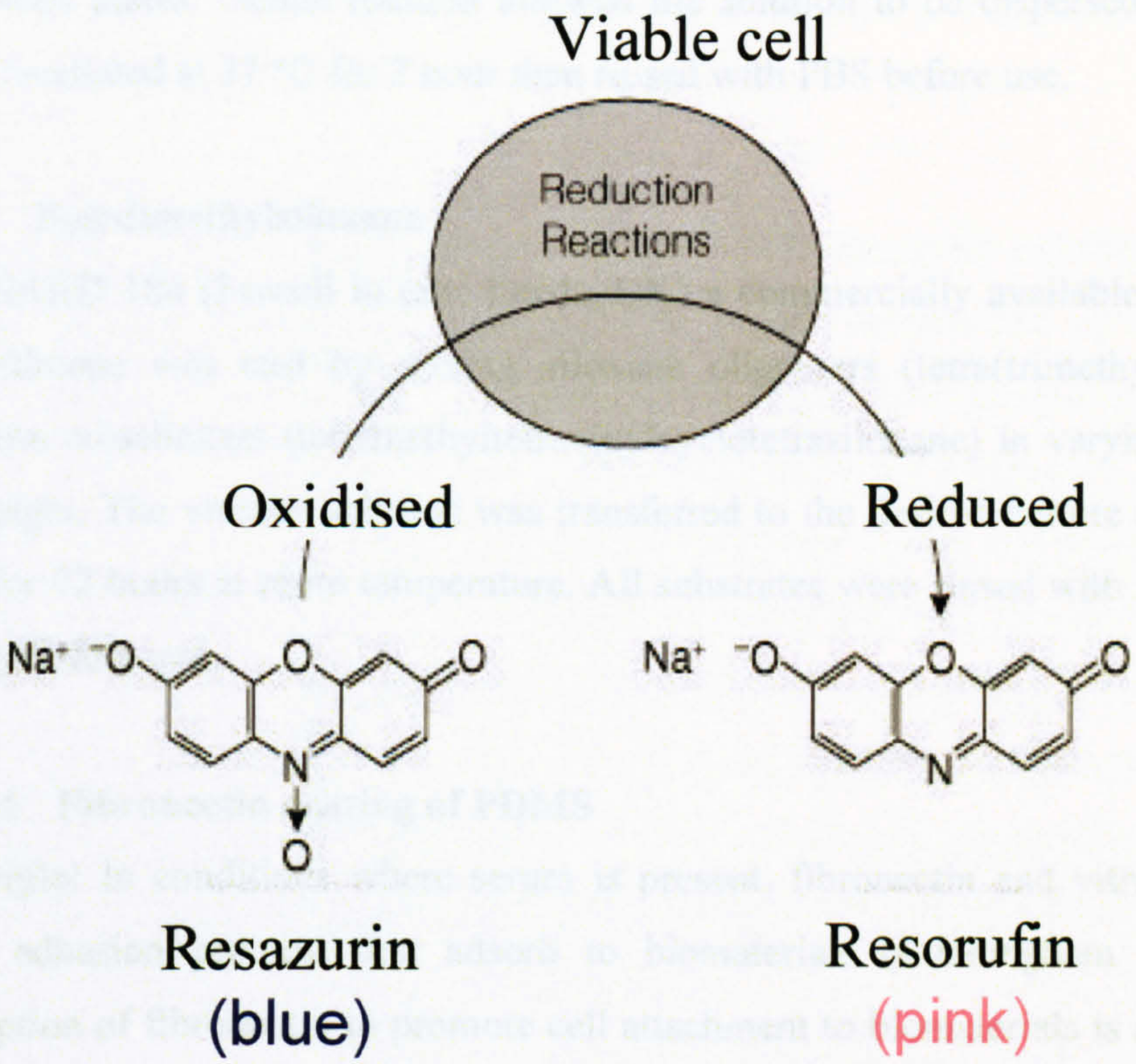


Figure 2-6: Alamar Blue™ is used to measure quantitatively the proliferation of cells. It works on the principal that Resazurin is reduced by metabolic activities of the cell to yield Resorufin. A colour change from blue to pink accompanies the reaction and can be detected colourimetrically at 630 nm (adapted from www.promega.com).

2.6 Substrate Preparation

Fibrin gels

Fibrinogen type I from bovine plasma was weighed and dissolved in serum free medium at 37 °C for a range of concentrations; 3-30 mg/ml. The mixture was gently agitated until dissolved, giving a hazy solution. The mixture was then cooled by refrigeration. Thrombin (1 unit/ml) at 4 °C (from bovine plasma) was added to induce gelation. For CFU-f cultures 5 ml of the solution was added to bacteria grade petri dishes (Greiner, Gloucester, UK). For secondary bone marrow stromal cell culture 300 µl of the solution was added per well into a 24 well plates. Gentle rotation allowed the solution to be dispersed evenly. The plates were incubated at 37 °C for 2 hour then rinsed with PBS before use.

2.6.1 Polydimethylsilaxane

SYLGARD 184 (Farnell in one, Leeds, UK) a commercially available kit was purchased. The silicone was cast by mixing siloxane oligomers (tetra(trimethylsiloxy)silane) and siloxane crosslinkers (tetramethyltetravinylcyclotetrasiloxane) in varying ratios 10:1-50:1, by weight. The viscous solution was transferred to the desired culture dish and allowed to cure for 72 hours at room temperature. All substrates were rinsed with PBS and allowed to air dry before use.

2.6.1.1 Fibronectin coating of PDMS

Principle: In conditions where serum is present, fibronectin and vitronectin are the two main adhesion proteins that adsorb to biomaterials (Cunningham et al., 2002). The adsorption of fibronectin to promote cell attachment to biomaterials is a well-characterised system and was therefore investigated for use as a possible coating to enhance cell attachment and subsequent growth to PDMS substrates.

Procedure: The dry lyophilized powdered protein was dissolved in PBS to an end concentration of 50 µg/ ml. 300 µl of the solution was added to each well of a PDMS coated 24-well plate and left for 1 hour at 37°. Prior to cell culture excess solution was removed.

2.6.1.2 Plasma polymerisation

Principle: Plasma polymerisation is a gaseous phase technique employed to coat pinhole free, nano-thin polymeric films (typically 10-100 nm) on to surfaces to enhance cell adhesion without compromising the bulk characteristics of the material. Plasma polymerisation is the transformation of low-molecular weight molecules ('monomer') into high-molecular weight molecules ('polymer'). The deposition occurs in a vessel containing the vapour of an organic compound under low pressure. The technique requires the supply of power, for example a radio frequency or microwave signal onto the desired substrate. This power is then matched in both phase and amplitude to the corresponding potentials in the plasma. The plasma polymer deposited is comprised of complex units from the initial polymer (Chu et al., 2002).

Procedure: For plasma deposition onto the PDMS substrates plasma polymerisation was employed whereby monomers are reacted in a plasma sustained by an electric field at radio-frequency. A schematic of the reactor used is shown in **Figure 2-7.** Built in house, the reactor consists of a stainless steel T-piece chamber (total volume 15.2 l). The chamber is closed at each end with aluminium flanges, secured by 3 bolts and sealed with PTFE O-ring gaskets. An inner power electrode is fed via a stainless steel rod from the supply unit. A two-stage rotary vane pump evacuates the chamber and a nitrogen filled cold trap, on the vacuum outlet of the chamber is used to trap unfragmented and fragmented monomer. Base pressure in the reactor was typically 9.6 $x10^{-2}$ mbar and approximately 3.1 x 10^{-2} mbar during plasma polymerisation. The leak rate, prior to addition of the monomer was on average less than 0.3 SSCM.

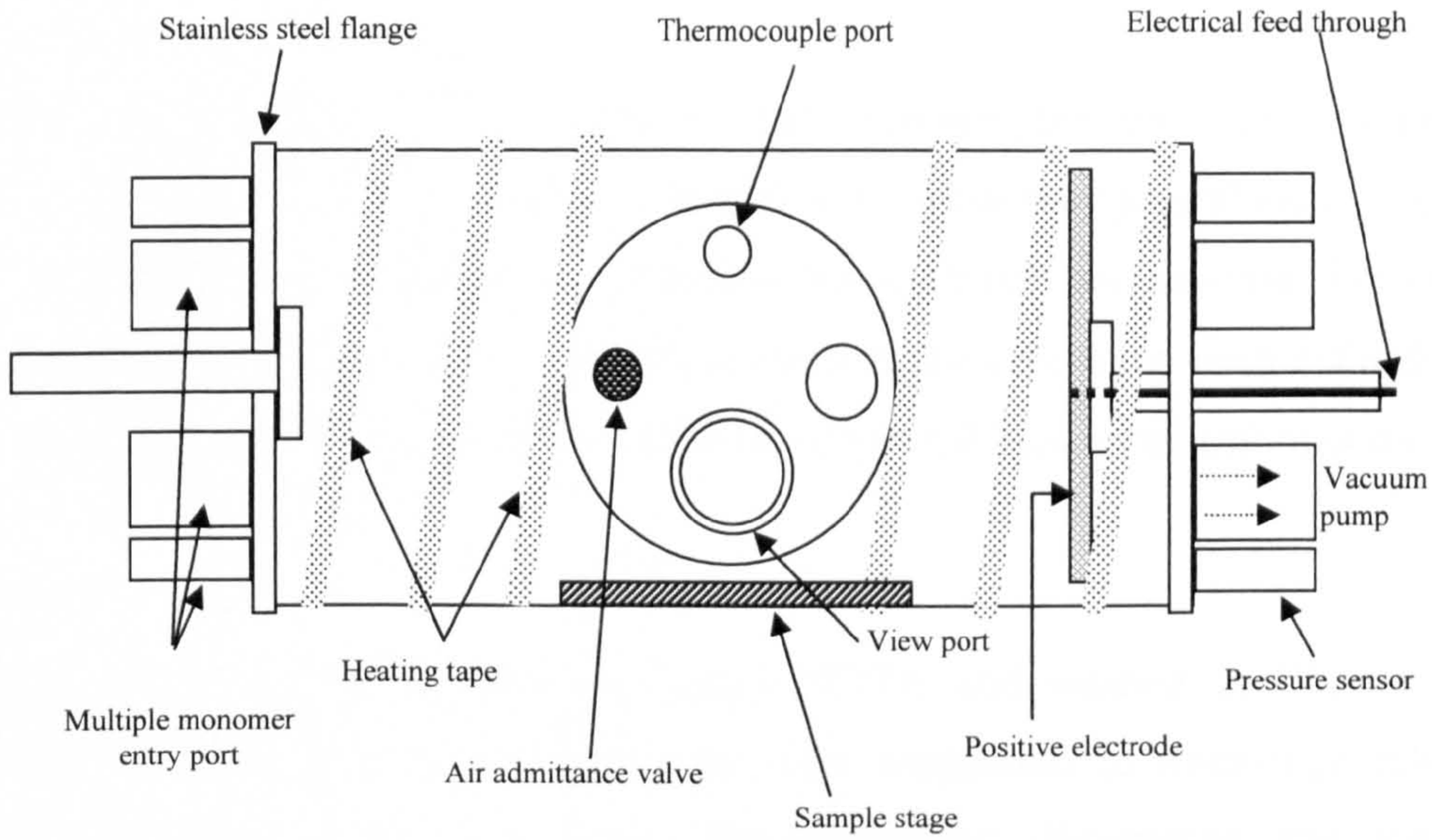


Figure 2-7: Schematic diagram of the RF plasma reactor (Salim et al., 2007).

Samples were placed in the central region of the chamber. Total flow rate was kept constant at 2 cm^3_{stp}/min. Flow rates were measured using the method of (Yasuda, 1985). Briefly, the vacuum pump was isolated for 30 seconds whilst the increase in reactor chamber pressure was measured using the pirani gauge a calculation was then completed to determine the flow rate (appendix one). The power was set at 10 W with the impedance matching unit adjusted for minimal reflected power; continuous monitoring ensured the reflected power did not change. The plasma was switched on for 10 minutes for deposition onto 55 cm^2 petri dishes and for 15 minutes when PDMS substrates within 24 well plates were being coated. Post-deposition monomer flow rates were measured before the flow was stopped and the reactor brought up to atmospheric pressure for the removal of the samples. Once the samples were removed they were wrapped in aluminium foil and kept at 4 °C until use. All samples were used within 72 hours.

2.7 Flow cytometry

2.7.1 Detection of CD antigens

Principle: Flow cytometry is a technique that measures the physical and chemical properties of cells. A laser beam is used to detect each cell as they pass alone in single file, with the information being directed to a computer for collection and analysis. Typically the light scatter enables cell size to be determined and if the cells have been stained with a fluorochrome against a protein of interest then additional biological information can be gathered.

Procedure: Cells were detached using trypsin/EDTA and washed in filtered growth medium (10 % serum). 5 x 10^5 cells were then transferred to microfuge tubes and centrifuged (2000 rpm) for 2 minutes, the supernatant disregarded and the pellet resuspended in 20 µl of the primary antibody (Serotec, Oxford, UK) (diluted as to the manufactures instructions). After incubation on ice for 30 minutes the cells were centrifuged (2000 rpm) 2 minutes and the supernatant removed. The cells were then washed with filtered medium and the pellet resuspended in 5 µl of PE-conjugated IgG, diluted 1:10 in filtered growth medium. After a further 20 minutes incubation at 4 °C, the cells were centrifuged, washed in filtered medium and resuspended in 500 µl of filtered medium for analysis using the Guava PCA.

2.7.2 Detection of apoptosis

Principle: During apoptosis, phosphatidylserine, a structural component of biological membranes, is translocated from the cytoplasmic face of the plasma membrane to the cell surface. Annexin V antibody has a strong affinity for phosphatidylserine and therefore is used as a marker for apoptosis.

Procedure: 5 x 10^5 cells were incubated with an annexin V antibody (Abcam, Cambridge, UK) conjugated to a phycoerythrin fluorochrome at a dilution of 1:1000, for 20 mins at room temperature. Cells were washed in filtered medium with 10 % serum and subsequently analysed in a personal flow cytometry system.

2.7.3 Cell cycle analysis

Principle: Information regarding the percentage of cells in a particular stage of the cell cycle is detectable using flow cytometry. The technique works on the principle that the DNA probes used are stoichiometric and consequently the amount of emitted light is proportional to the amount of bound probe and therefore DNA. During replication the cell doubles its DNA content, before splitting to form two identical cells. Measuring the amount of emitted light can therefore distinguish between cells in the different phases of the cell cycle. The G0/G1 peak represents cells that are quiescent and the cells that will divide but have not begun to replicate their DNA (**Figure 2-8**).

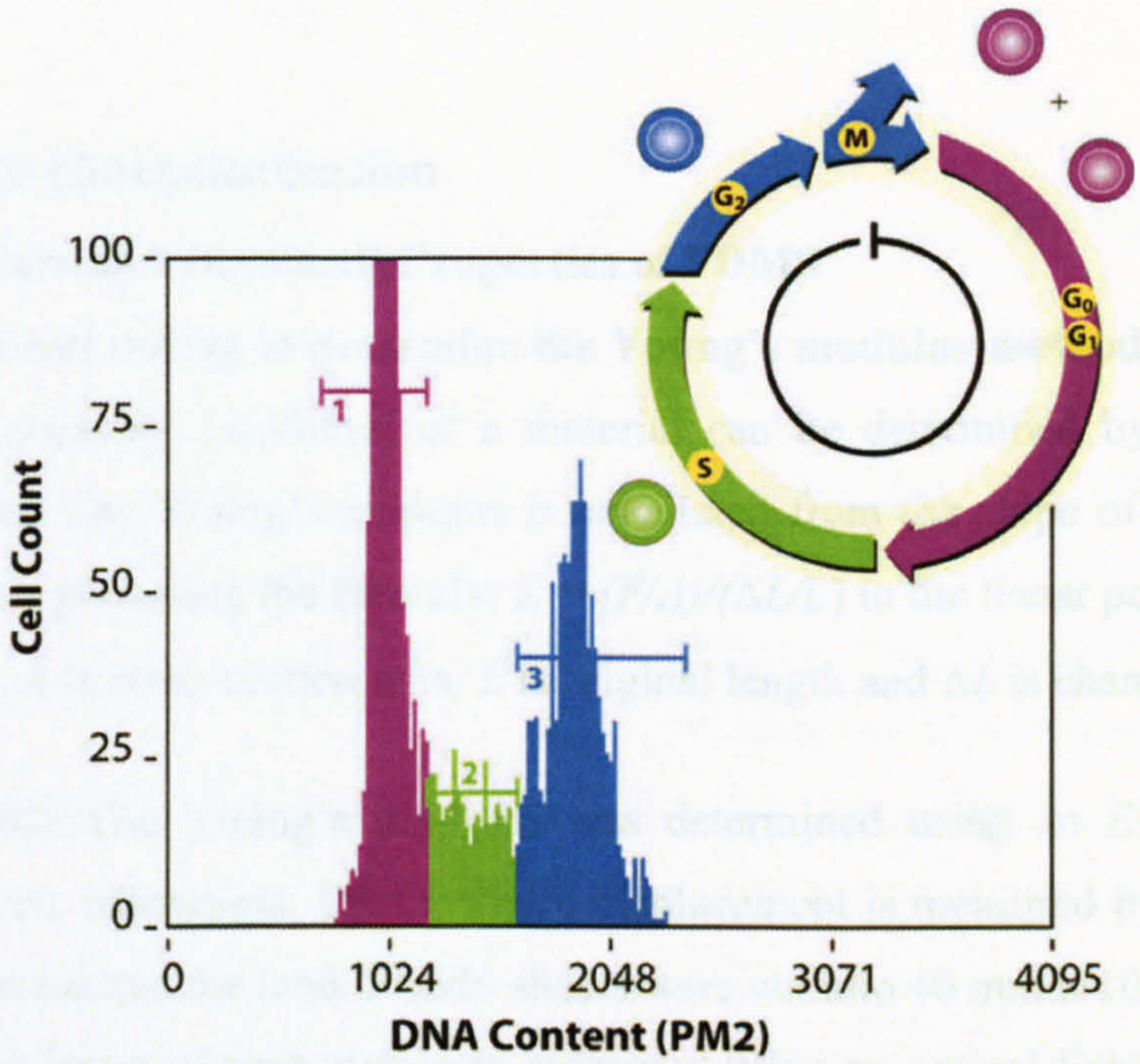


Figure 2-8: A representation of a typical graph obtained using flow cytometry for the analysis of the cell cycle. The percentage of the cell population in G_0/G_1, S and G_2/M can be determined by staining the DNA and then measuring the amount present within the cell (adapted from www.biosciencetechnology.com).

Procedure: Cells were trypsinised, washed with PBS and fixed in ice cold IMS (70%) for at least 12 hours. The cells were then transferred to microcentrifuge tubes (Fischer Scientific, Pennsylvania, USA) and pelleted (2000 rpm for 2 mins), and washed with PBS before excess staining solution (95.5 % 0.1 % Triton X-100, 2.5 % propidium iodide (1

mg/ml) and 2 % DNAse-free RNAse in distilled H_2O (10 mg/ml)) was added. Cells were gently mixed and then incubated for 30 mins at room temperature in the dark. Samples were read on the Guava PCA with typically 5000 events being acquired.

2.7.4 Cell size analysis

Changes in cell size by measuring forward scatter flow cytometrically has been previously used to demonstrate differences in cell size with differing culture conditions (Sekiya et al., 2002). The average forward scatter was calculated using "cytosoft" software with 1000 cells assayed for each sample.

2.8 Substrate characterization

2.8.1 Characterising Viscoelastic Properties of PDMS

2.8.1.1 Mechanical testing to determine the Young's modulus-method one

Principle: The elasticity (stiffness) of a material can be determined by calculating the Young's modulus. The Young's modulus is calculated from the slope of a strain *(ΔL/L)* versus stress *(F/A)* plot using the formula; $E = (F/A)/(\Delta L/L)$ in the linear portion of the plot, where F is force, A is cross-section area, L is original length and ΔL is change in length.

Procedure: PDMS-The Young's modulus was determined using an Electroforce 3200 tensile tester (Bose, Minnesota, USA), where displacement is measured by a LVDT and a 450 N load cell measures the load. PDMS sheets were cut into 40 mm x 10 mm strips using a scalpel. The thickness of each strip was measured using an optical light microscope, (to ascertain cross sectional area). Each end of the test strip was protected with 10 mm autoclave tape, giving a test length of 20 mm. The test was performed at a rate of 0.1 mm/s until the instrument's limit was reached at 6 mm. Data was recorded to give a load / displacement curve. Stress strain plots were obtained in the linear region typically between 2 mm and 4 mm displacement. All experimentation was performed at room temperature.

Fibrin- The stiffness was determined using an Electroforce 3200 (Bose, Minnesota, USA). Fibrin gels were cut into 1 cm^3 using a scalpel. The compression test was performed up to a displacement of 2.5 mm at 0.1 mm s^{-1}. A stress / strain plot was obtained between 1.8-2.2

cm displacement. For fibrin there was no linear region, on the plot, therefore stiffness was calculated using a tangent modulus taken at 20 % strain (Callister, 2000).

2.8.1.2 Mechanical testing to determine the Young's modulus-method two

Principle: To characterise the viscoelastic properties of the PDMS substrates a novel experimental technique was employed. The technique measures the central indention of a circumferentially clamped membrane after the addition of a stainless steel ball of known weight. The theory behind the technique is that a film which has elastic modulus *E,* an un-deformed radius *a* and thickness *h,* will have a displacement δ occur at the pole when a central load *w* is applied via a sphere of radius *R* as shown in

Figure 2-10. A non-linear analyses method developed by Yang, (1971) can then be used to determine the elastic modulus.

Procedure: 300 µl of PDMS was cast in a ring of filter paper with inner diameter of 20 mm and allowed to cure for 72 hours. A specifically designed sample holder in which the sample was held between two transparent plastic discs of inner diameter 20 mm clamped the samples around their outer circumference. Two thin metal sheets were then used to clamp the discs together by a number of stainless steal screws. Once securely in place the PDMS sample was immersed in PBS and indented with a stainless steal ball of weight 0.27 g and diameter 4 mm. Deformation was then recorded laterally by means of a long distance microscope and digital camera apparatus. The time of measurement after deformation was kept constant for each sample. Deformation lengths were calculated using image analysis using Paint (Microsoft, USA).

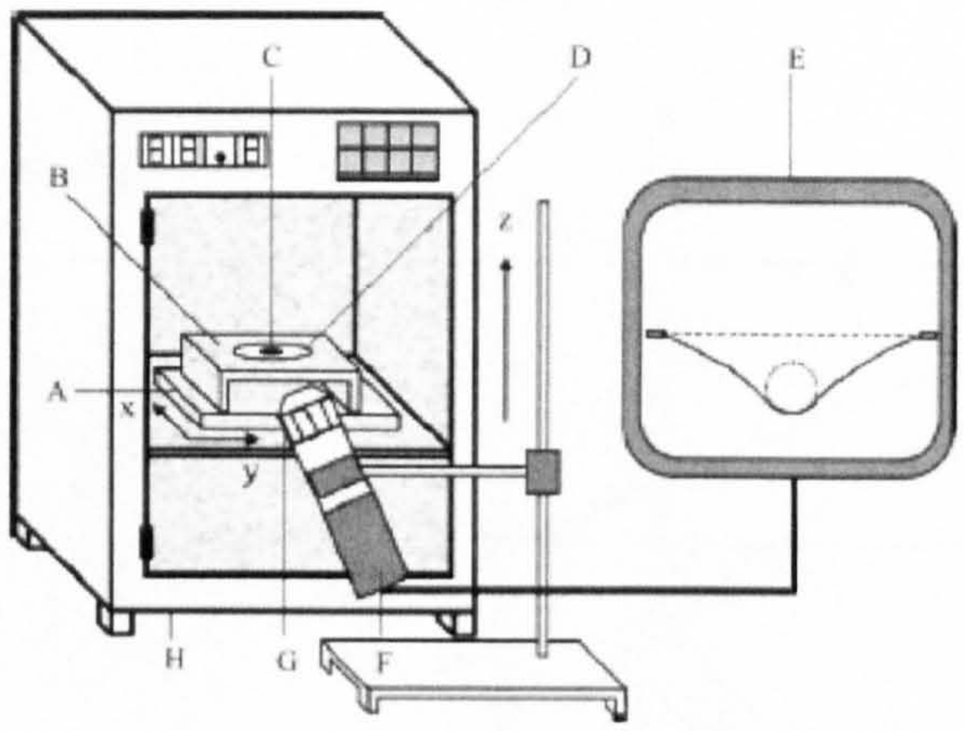


Figure 2-9: A schematic representation of the instrument system used for mechanical testing. It comprises a precision two-dimensional (X-Y) translation stage (A), aluminium plate (B), an oil-lubricated stainless sphere of specific size and mass (C), circular membrane (D). The core of the instrument is a computerised microscope-enhanced image system (F) which comprises a long focal distance objective lens (G) incorporated with computer-linked CCD camera (E). The instrumentation is housed within a constant temperature and humidity chamber (H) (adapted from Ju, 2002).

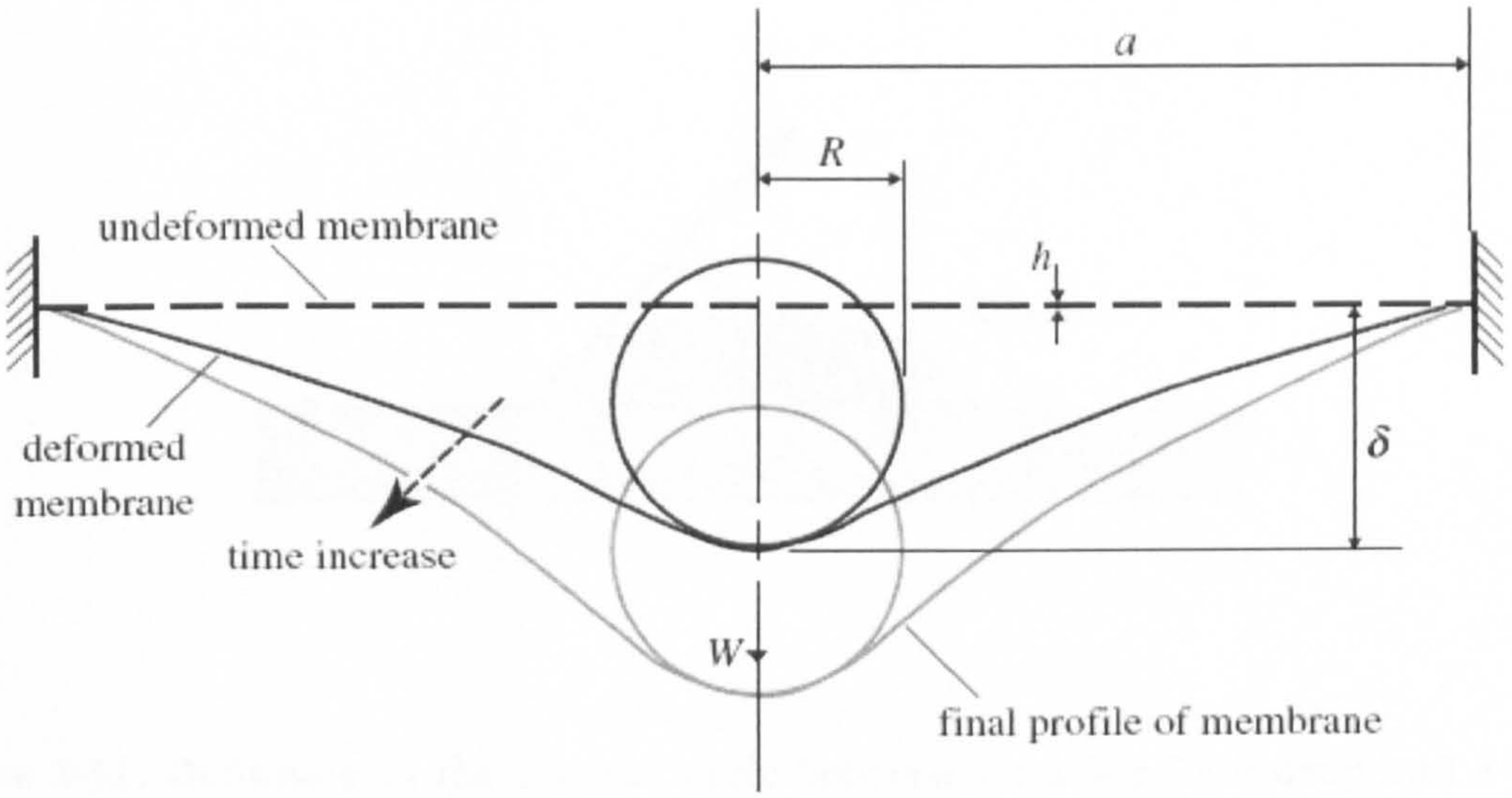


Figure 2-10: Indentation of a circular membrane by a smooth sphere (adapted from Ahearne., et al 2005). The technique measures the central indention of a circumferentially clamped membrane after the addition of a stainless steel ball of known weight, *w*. The theory is that a film which has elastic modulus *E*, an un-deformed radius *a* and thickness *h*, will have a displacement δ occur at the pole when a central load *w* is applied via a sphere of radius *R*.

2.8.2 Contact Angle Measurements

Principle: A visualization method to determine the surface energy of a material. The contact angle is the angle of the interface at which a liquid drop meets a solid surface. The angle can be used to gain information relating to the surface chemistry and chemical bonding of the underlying material.

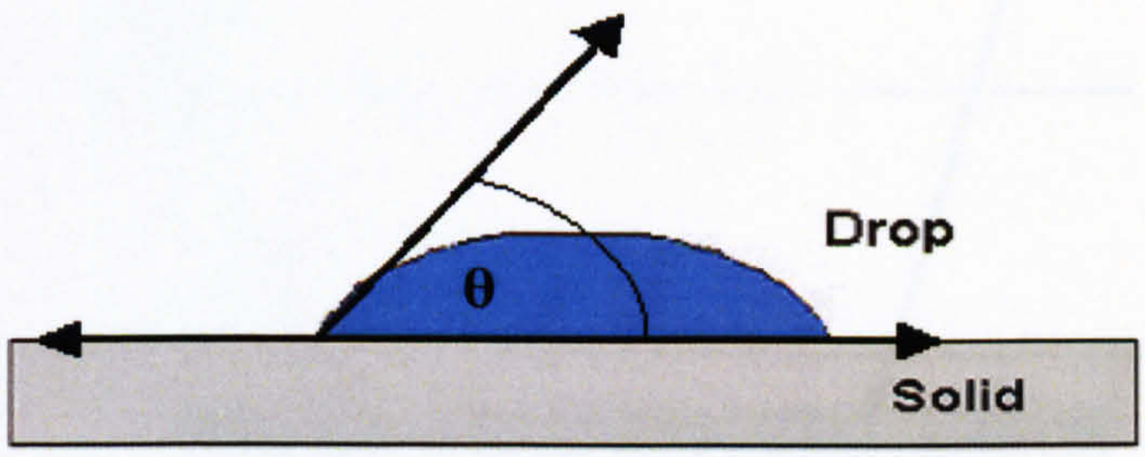


Figure 2-11: Definition of the contact angle between a resting fluid drop and a solid surface.

Procedure: Contact angle measurements were measured by sessile drop method using a goniometer. Contact angles for 5 different locations of treated and untreated PDMS samples were measured. The contact angle for each condition was calculated as the average measurements from 2 independent samples.

2.8.3 X-ray photoelectron spectroscopy

Principle: X-ray photoelectron spectroscopy is a surface analysis technique employed for the identification and characterisation of the surface chemistry, typically the first 5-10 nm, of a material. The technique analyses the elemental composition of the surface and subsequent analysis of the resultant spectra enable chemical structures to be determined. The technique works on the principal that the absorption of x-ray energy by the core level electrons results in the ejection of core electrons (photoelectron) from the atoms **(Figure 2-12)**. The energies from the ejected photoelectrons are unique to each element and therefore enable elemental composition of the surface to be determined. Examination of the obtained

spectra enables elemental concentration to be calculated (as the intensity of the photoelectrons peak produced is proportional to the elemental concentration) and gives information on chemical states. All elements with the exception of H and He are deflected.

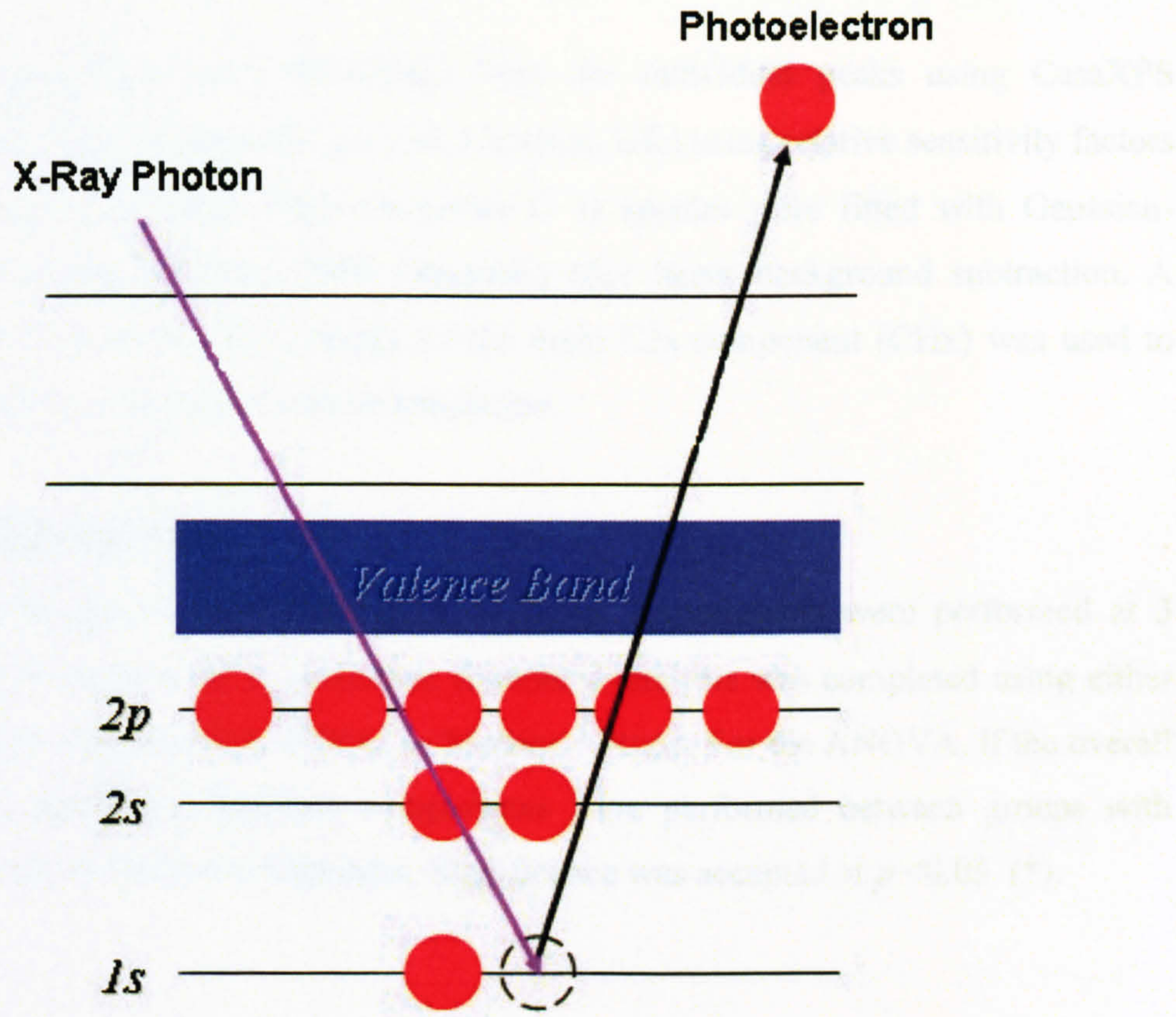


Figure 2-12: The physical basis of XPS: emission of a photoelectron from the core level after excitation by an X-Ray photon (adapted from http://www.ifw-dresden.de).

Procedure: XPS was carried out on a Kratos Ultra DLD photoelectron spectrometer (Kratos Analytical Ltd, Manchester, UK) equipped with a monchromated Al Kα source at a power of 150 W (300x700 μm spot) operating in the hybrid lens mode. The pressure in the main UHV chamber was maintained below 5 x 10-8 mbar for all analyses. All data were collected at 90 °C relative to the sample plane giving an analysis depth of ~10 nm (Briggs and Seah., 1990). Three survey scans were performed on each of the samples at pass

energies of 160 eV and step of 1 eV to identify and quantify the elements present. High resolution C1s spectra were also obtained from one point on the sample at 20 eV and step width of 0.1 eV. An electron flood gun was used at all times to charge neutralise the samples.

Elemental compositions were determined from the individual peaks using CasaXPS software version 2.3.10 (Casa Software Ltd, Cheshire, UK) using relative sensitivity factors supplied with the instrument. High resolution C 1s spectra were fitted with Gaussian-broadened Lorentzian functions (80% Gaussian) after linear background subtraction. A value of 285 eV for the binding energy of the main C1s component (CHx) was used to correct for charging of specimens under irradiation.

2.9 Statistical analysis

Values are expressed as mean ± standard deviation. Experiments were performed at 3 independent time points with 3 repetitions. Statistical analysis was completed using either one-way analysis of variance (ANOVA) or Student's *t*-tests. For the ANOVA, if the overall difference was significant, multiple comparisons were performed between groups with Tukey's test using the software Sigmastat. Significance was accepted at $p < 0.05$ (*).

CHAPTER THREE: The use of fibrin as a flexible cell- culture substratum

3.1 Introduction

MSC isolated from bone marrow have received much attention since they were first described by Friedenstein over 30 years ago (Friedenstein et al., 1974). The ease of isolation, rapid proliferation and well defined multilineage differentiation capacity into a variety of tissues including bone, cartilage and fat (Pittenger et al., 1999) makes them a strong candidate for use in gene and cell therapy (Jorgensen et al., 2004). The availability of autologous cells from a clinically accessible site, that can be easily expanded and manipulated in culture before reintroduction in an engineered scaffold, therefore makes them particularly attractive in the field of tissue engineering.

In vitro analysis has determined that only a very small fraction, approximately 0.001-0.01%, of the total population of nucleated cells obtained from bone marrow are plastic adherent, with a capability for clonogenic replication and multipotent differentiation capacity (Pittenger et al., 1999). With approximately 10^9 cells needed for therapeutic applications (Chen et al., 2004), exponential growth of the cells in culture is required. Extensive culture of MSC is also hindered by their limited proliferative life span (Banfi et al., 2002) (Stenderup et al., 2003) due to telomere shortening (Banfi et al., 2002), and accumulation of senescent cells (DiGirolamo et al., 1999). Loss of differentiation potential is also seen in culture with age (Scutt and Bertram, 1995). Furthermore, decreased proliferation potential and accelerated senescence is exhibited in cells obtained from elderly donors compared to younger donors (Stenderup et al., 2003).

Bruder et al., (1998) highlighted the potential use of MSC in tissue engineering when MSC were combined with biomaterials to repair a large femur defect in a rat model. Since then a number of animal studies have demonstrated the potential of MSC in a clinical setting but despite this, there is no well-defined protocol for isolation and expansion of MSC. Culture conditions which allows for exponential growth but with full retention of the multilineage differentiation potential need to be defined to enable the use of MSC in regenerative medicine. Preservation of differential potential in culture overtime would reduce the number of cells needed, thereby, enabling cell culture for tissue engineering applications to be quicker and cheaper. A consensus on methodologies would also allow for better comparison and extrapolation of results between different research groups.

At present expansion of MSC involves a density gradient centrifugation and plastic adherence selection before culture on tissue culture plastic in serum rich medium (Gregory et al., 2005). Investigations into the expansion of MSC are typically performed on tissue culture plastic, a hard substrate in contrast to their naturally soft natural microenvironment. Most cells *in vivo* reside in a relatively elastic microenvironment with attachments to other cells and/or the extracellular matrix (Discher et al., 2005).

Recent work has shown that substrate stiffness affects a number of cellular functions including migration (Pelham and Wang, 1997), apoptosis (Wang et al., 2000) and differentiation (Engler et al., 2004b). Although an exact mechanism is still to be elucidated it is believed that integrin binding induces focal adhesions which then link the ECM to the internal environment of the cell via the actin cytoskeleton (Pelham and Wang, 1997). Through this connection and with the recruitment of a number of adhesion proteins a number of signalling pathways are induced leading to changes in the cells behaviour (Gaudet et al., 2003). Previous reports have already shown the importance of basement membrane ECM in the growth and retention of differential potential in MSC (Matsubara et al., 2004).

In the study, we employ fibrin as a natural substrate in the study of the effects of substrate stiffness on the growth of MSC. Mechanical testing was completed to characterise the system and then two approaches were taken to evaluate the effects of the substrate on cellular behaviour. First, cells were cultured in direct contact with the fibrin gels and then secondly, cells were selected and pre-cultured on the fibrin gels before analysis on TCP. A number of cell-based assays were then used to determine if cell behaviour is altered by the underlying substratum with emphasis on the differentiation potential.

3.2 Materials and methods

Refer to chapter two.

3.3 Results

3.3.1 Fibrin gel preparation

Fibrin gels were made by the addition of thrombin to a solution of fibrinogen dissolved in serum free medium. Serum free medium is used to prevent premature clotting catalysed by factors present in the serum. If the fibrinogen solution is cooled sufficiently, the mixture can be transferred to the desired culture dish and gentle agitation performed to allow even coverage before gelation occurs.

Simply altering the amount of fibrinogen dissolved into the medium was enough to change the stiffness and hence the elastic properties of the resultant gel.

Fibrinogen was used at concentrations of 3, 10, 30 mg/ml as previous research has shown that these concentrations cover the range of attainable elastic moduli (Brouwers., 2002). The concentration of thrombin was kept constant at 1 unit per ml. A fibrinogen concentration lower than 3 mg/ml created gels that were not solid enough to be handled properly and a minimal concentration of 3 mg/ml could be used to ensure that the gel did not dissolve in culture over time. Macroscopic observations evaluated by visual inspection confirmed that there was indeed no detectable degradation over time in culture. For use as a cell culture substratum, it was found that two hours at 37 °C was adequate time to ensure complete gelation, prior to the cells being seeded.

3.3.2 Mechanical testing of fibrin gels

The stiffness of the fibrin gels was determined by compression testing using an Electroforce 3200 (Bose, Minnesota, USA). A stress strain plot obtained revealed that there was no linear region and therefore the stiffness was measured using a tangent modulus at 20 % strain. The data showed that as the concentration of fibrinogen increased so did the stiffness with a modulus of 16.7 kPa for 3 mg/ml, 40.4 kPa for 10mg/ml and 77 kPa mg/ml for 30 mg/ml. The stiffness of the 30 mg/ml was statistically different from the 3 and 10 mg/ml.

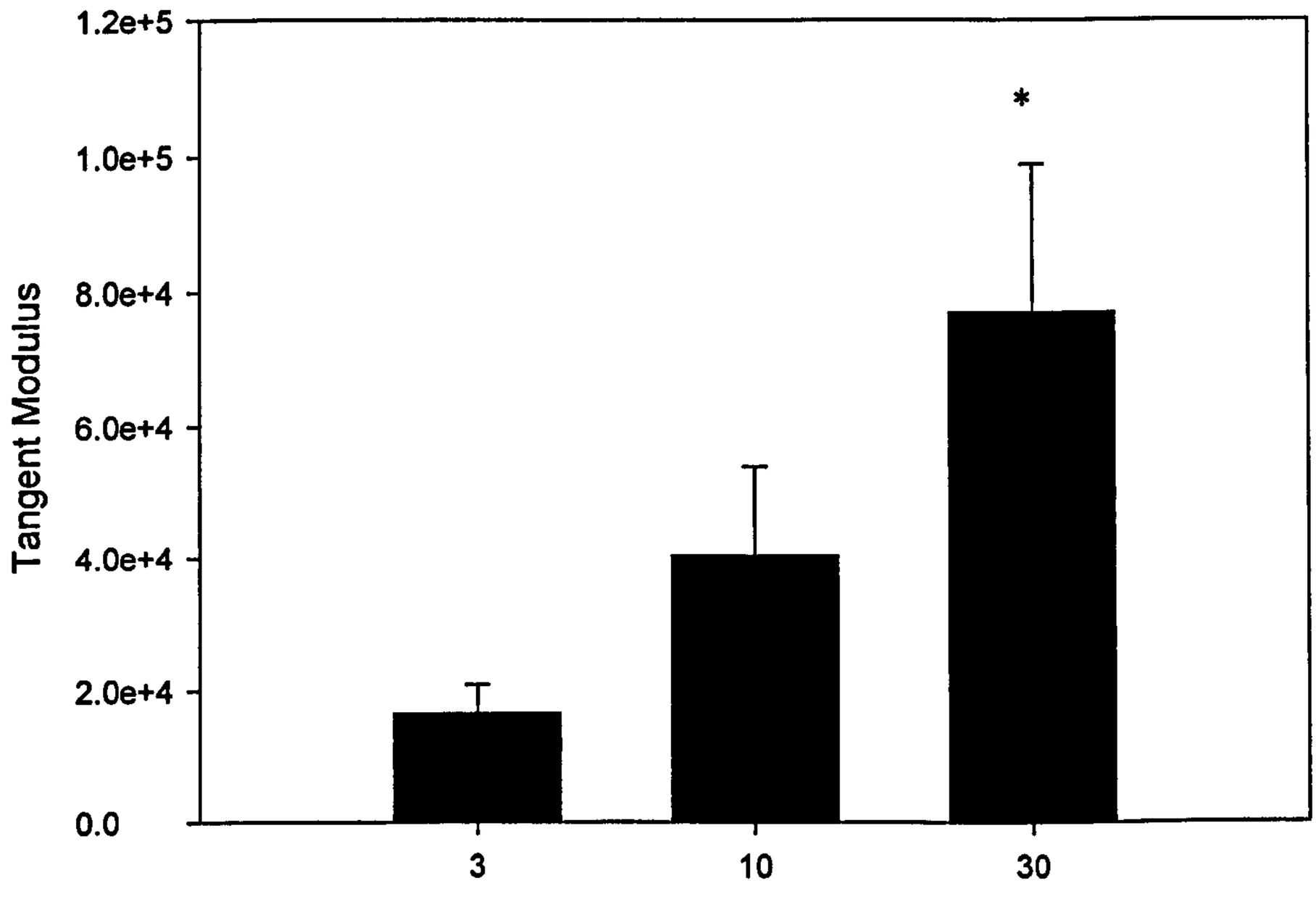


Figure 3-1: Tangent modulus of a range of fibrin gel produced with varying fibrinogen concentrations. Fibrin gels were made by mixing fibrinogen (3, 10 and 30 mg/ml) with thrombin (1 U/ml). Tensile testing of the gels was performed using a Bose Electroforce 3200. The tangent moduli were calculated from the slope of the plot of stress versus strain at 20 % strain for 6 different samples. * denotes a statistically significant difference from the other groups.

3.3.3 The effect of fibrin gel concentration on the expansion of MSC

3.3.3.1 Alamar blue

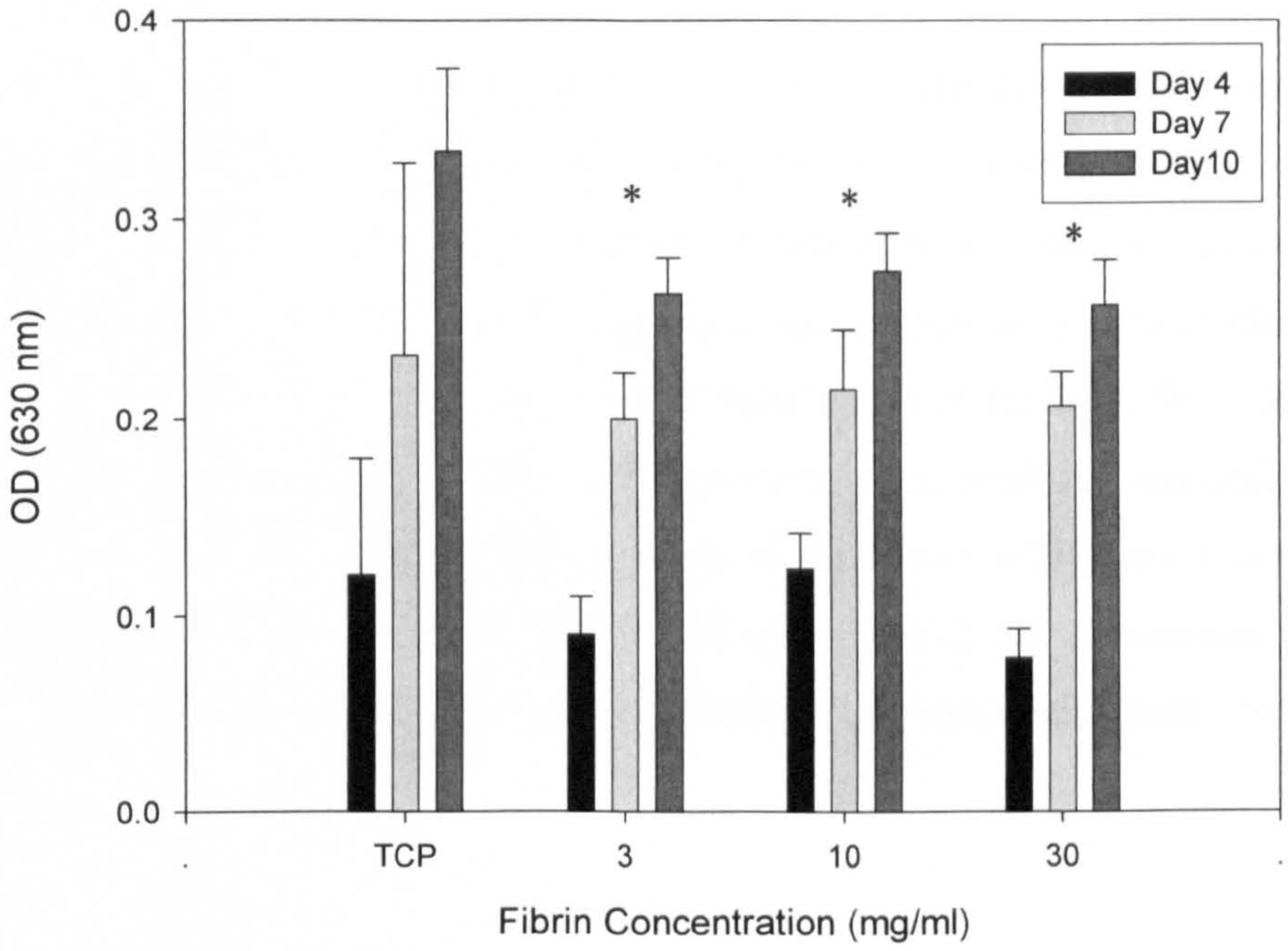


Figure 3-2: Cell proliferation of bone marrow derived MSC on fibrin substrates. 2 x 10^4 MSC were cultured on fibrin gels at concentration of 3, 10 and 30 mg/ml and a TCP control. Cell proliferation rates were determined using the Alamar blue assay on day 4, 7 and 10, (N=6). * denotes statistically significant from corresponding TCP control.

Primarily, the alamar blue assay was employed to determine MSC proliferation rates on a range of fibrin concentrations (3, 10 and 30 mg/ml) and a TCP control. Optical density readings at 630 nm were taken on day 4, 7 and 10. Although the cells were seen to proliferate over this time frame, readings did not match visual observation and the effectiveness of the assay was questioned **(Figure 3-2).** Fibrin is a porous gel, which therefore absorbs any liquid present. The alamar blue assay works by the addition of a colorimetric growth indicator that is biochemically reduced by mitochondrial metabolic activity. The dye is supplemented to the cell's medium as a 1:10 dilution and although background readings were taken into consideration, it was found that the medium contained within the fibrin altered this precise dilution and therefore interfered with the readings

taken by changing the optical density of the resultant solution. Even results between the different fibrin groups were not comparable due to differences in background readings caused by the differences in the gels and protein concentration.

Due to the porous nature of the fibrin gels it was decided that the most accurate method of determining cell proliferation was by trypsinising the cells and subsequent counting of the cells. Detaching cells from a surface is usually achieved by the addition of trypsin/EDTA solution at a concentration of <1 %. Trypsin is a serine protease and is inactivated by the serum present in cell culture medium. For the detachment of the cells from the fibrin, the gels were washed three times with PBS and then serum free medium was added for 1 hour prior to trypsinisation to allow for the removal of the serum within the fibrin gels. Cells were then detached using a slightly higher level of trypsin (2 %). Percentage cell viability was measured to ensure that the cells were not affected by this detachment process (**Figure 3-4**).

3.3.4 Growth curve

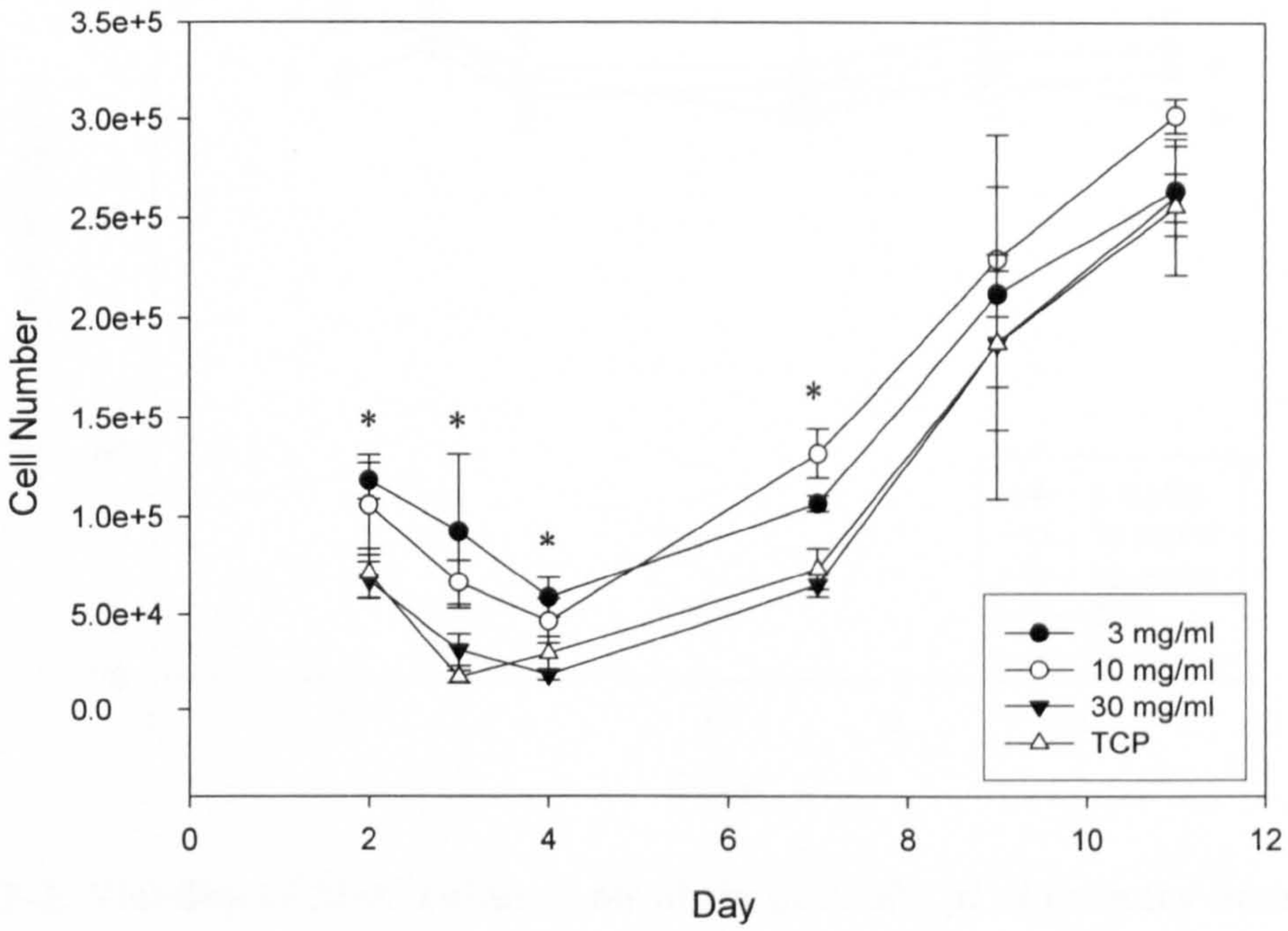


Figure 3-3: Growth curve of MSC on a range of fibrin gels (3, 10, 30 mg/ml) and TCP. 250 μl of primary bone marrow was cultured on a range of fibrin gels and a TCP control in growth medium. Cell number was determined on day 2, 4, 7, 9 and 11 by detachment of the cells and subsequent counting using a Guava PCA. Data are shown as the mean of three independent experiments ± SD (n=3). (For statistical analysis see appendix two).

Once detached from the fibrin and in suspension, cell number was determined using a Guava PCA. A growth curve over an 11-day period was completed to evaluate the growth of MSC on a range of fibrin concentrations (3, 10 and 30 mg/ml); TCP was used as a control. 300 μl of primary bone marrow cells were used as previous work had shown that this volume was sufficient to provide a confluent monolayer that was sustainable in culture for 14 days. Cells were seen to expand better on the 3 and 10 mg/ml compared to the 30 mg/ml and TCP control although by day 9 this difference was not significant (**Figure 3-3**).

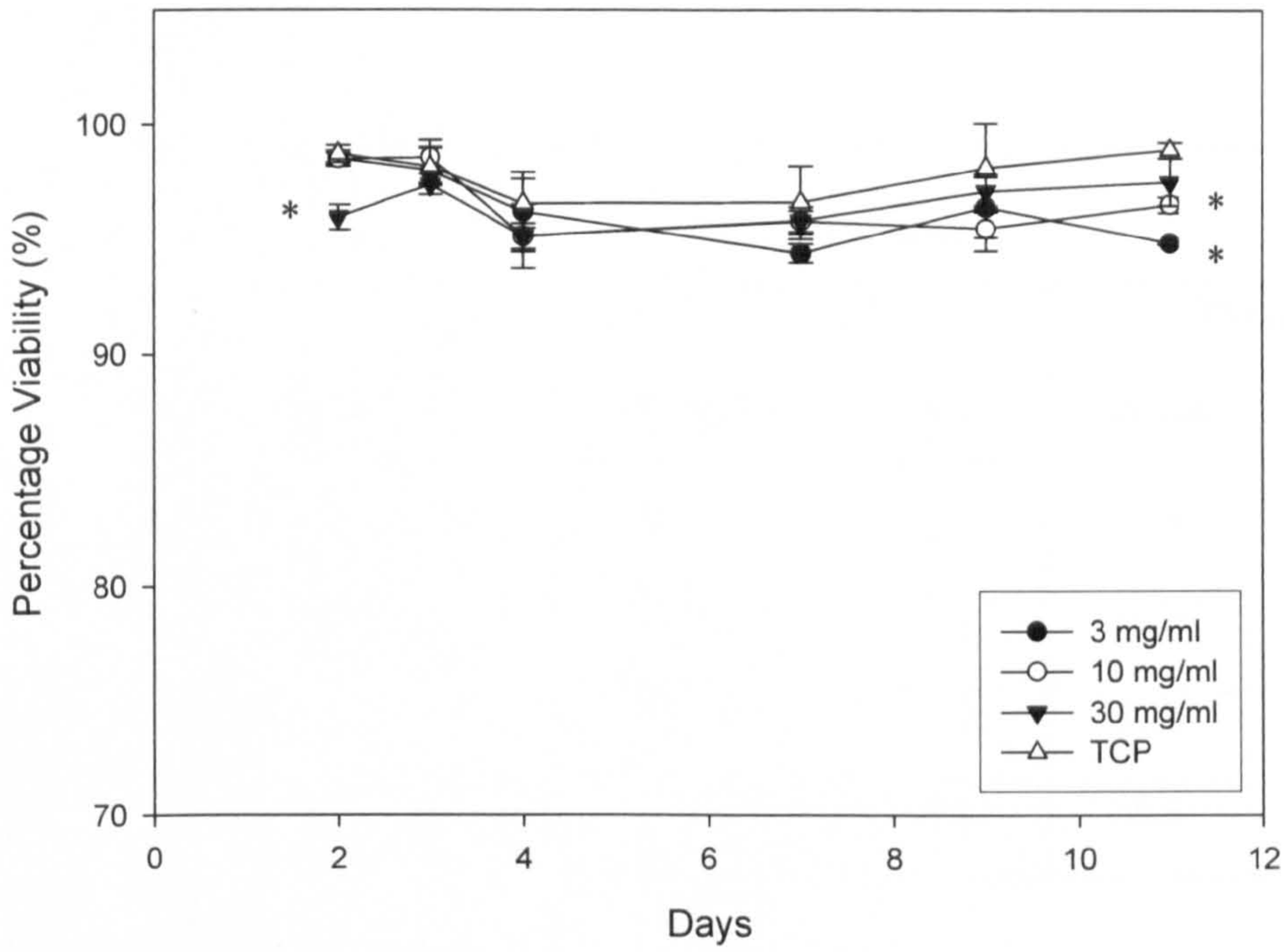


Figure 3-4: Viability of MSC cultured on fibrin gels. 300 µl of primary bone marrow cells were cultured on fibrin gels with varying concentrations and a TCP control. Cell viability was measured on the detached cells on day 2, 4, 7, 9 and 11 using Guava PCA Viacount reagent and subsequent analysis in the Guava PCA. Data are shown as the mean of three independent experiments ± SD (n=3). (For statistical analysis see appendix two).

Cell viability was assessed over an 11 day time period for cells cultured on fibrin gels (3, 10 and 30 mg/ml) and a TCP control. The percentage of viable cells was maintained between 94.8 % and 98.8 % regardless of the substrate, for the duration of the culture. The cells cultured on the 30 mg/ml were slight lower (statistically significant) on day 2 but this difference was need seen at any other time point. The cells on the 3 and 10 mg/ml fibrin concentrations were also seen to be statistically lower towards the end of the culture period, and was probably due the cells becoming confluent and overcrowded. Cell on the TCP control maintained the highest level of viability throughout the time period with a percentage viability never dropping below 96.5 %.

3.3.5 The effects of culturing MSC on fibrin gels

3.3.5.1 Apoptosis levels

		Experiment					
		One		Two		Three	
		Adherent	Non-adherent	Adherent	Non-adherent	Adherent	Non-adherent
Substrate	TCP	13.9	5.7	0.1	0.3	12.7	9
	Fibrin (mg/ml) 3	16.1	5.3	0.1	1.3	18.0	24.9
	Fibrin (mg/ml) 10	11.3	8.2	0.2	1.3	21.8	33.6
	Fibrin (mg/ml) 30	18.1	10.4	0.9	9.4	26.4	23.7

Table 3-1: Effects of culture on fibrin cells on apoptosis levels in cultured MSC. 2 ml of primary MSC were plated out per 25-cm^2 well on fibrin substrates (3, 10 and 30 mg/ml) and a TCP control in growth medium and cultured at 37 °C for 7 days. Apoptosis levels were ascertained by the positive staining for Annexin V of both the attached and non-adherent populations using FACS and data expressed as percent positive relative to the whole population (2000 events were read per sample). Data is presented for three independent experiments (n=1).

To confirm the cell viability data, which measures the percentage of live to dead cells, apoptosis levels were also measured to determine if any of the cells had initiated the apoptosis pathway. Apoptosis levels were ascertained by measuring Annexin V levels using flow cytometry on day 7 of culture, as the cells reached 80 % confluency and were typically sub-cultured and used for experimental purposes. Levels were measured in the adherent cell layer but also in the non-adherent cell fraction to record the number of cells that had entered apoptosis and consequently detached from the substrate (**Table 3-1**).

For both the adherent and non-adherent fractions the levels of apoptosis typically increased as the fibrin concentration increased from 3-30 mg/ml. The lowest levels were also typically seen on the TCP control.

3.3.5.2 MSC attachment

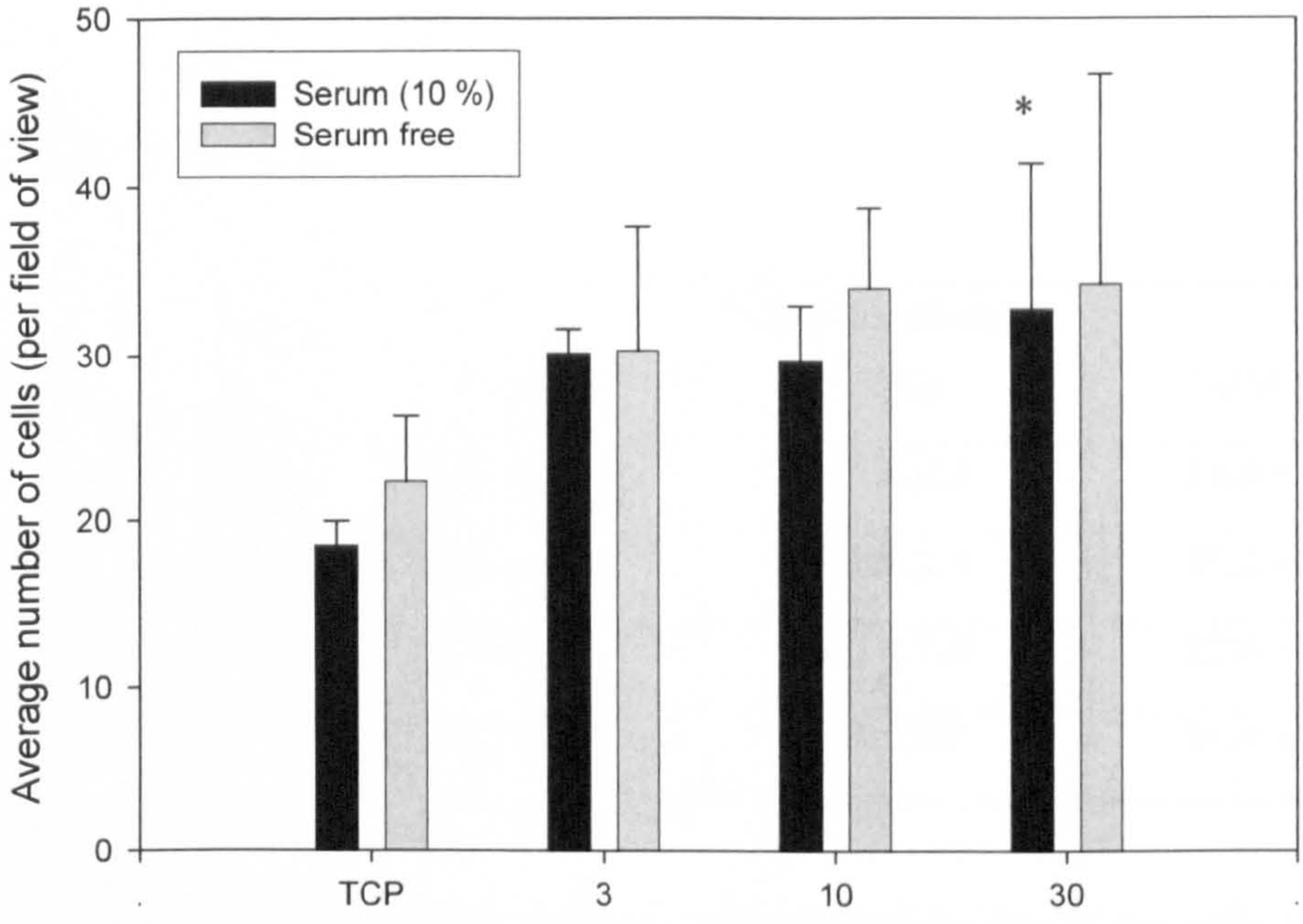


Figure 3-5: Quantification of MSC attachment to fibrin gels and TCP after 24 hours. 2×10^5 MSC were seeded onto the fibrin gel substrates in 2-cm well plates coated with different fibrinogen concentrations (3, 10 and 30 mg/ml) and a TCP control and cultured in either serum rich or serum free growth medium at 37 °C. After 24 hours the substrates were washed, cells fixed and stained with DAPI before subsequent counting. Numbers are expressed as cells counted per field of view, with 5 fields of view per well and 3 wells counted for each substrate type (n=3). * denotes significant difference from corresponding TCP control.

After 24 hours cell attachment was seen to be higher on the fibrin gels compared to the TCP control, although only significantly so for the 30 mg/ml. No significant difference was seen between the fibrin gels with an average of 31 cells attaching per field of view compared to 18 per field of view for TCP. No statistical significant difference was seen between attachment of the MSC in serum rich and serum free medium (**Figure 3-5**).

3.3.5.3 Cell cycle analysis

		Cell cycle analysis		
		G_0/G_1 (%)	S (%)	G_2/M (%)
Substrate	TCP	82.5 ± 6.4	3.7 ± 0.3	13.8 ± 6.7
Fibrin (mg/ml)	3	69.7 ± 4.6	5.1 ± 1.4	25.2 ± 3.2
	10	72.5 ± 2.5	4.7 ± 1.5	22.8 ± 3.2
	30	72.3 ± 5.1	6.3 ± 3.2	21.4 ± 2.5

Table 3-2: Cell cycle distribution analysis determined by FACS for cells cultured on a range of fibrin substrates (3-30 mg/ml) and a TCP control. Data are shown as mean ± SD of three independent experiments.

There was no significant difference in the cell cycle distribution analysis, determined by FACS analysis, for cells cultured on a range of fibrin substrates (3-30 mg/ml) and a TCP control. The majority of cells were consistently seen in G_0/G_1 phase with approximately 75 % of the population. An average of 5 % and 21 % were seen in S phase and G_2/M respectively (**Figure 3-6** and **Table 3-2**).

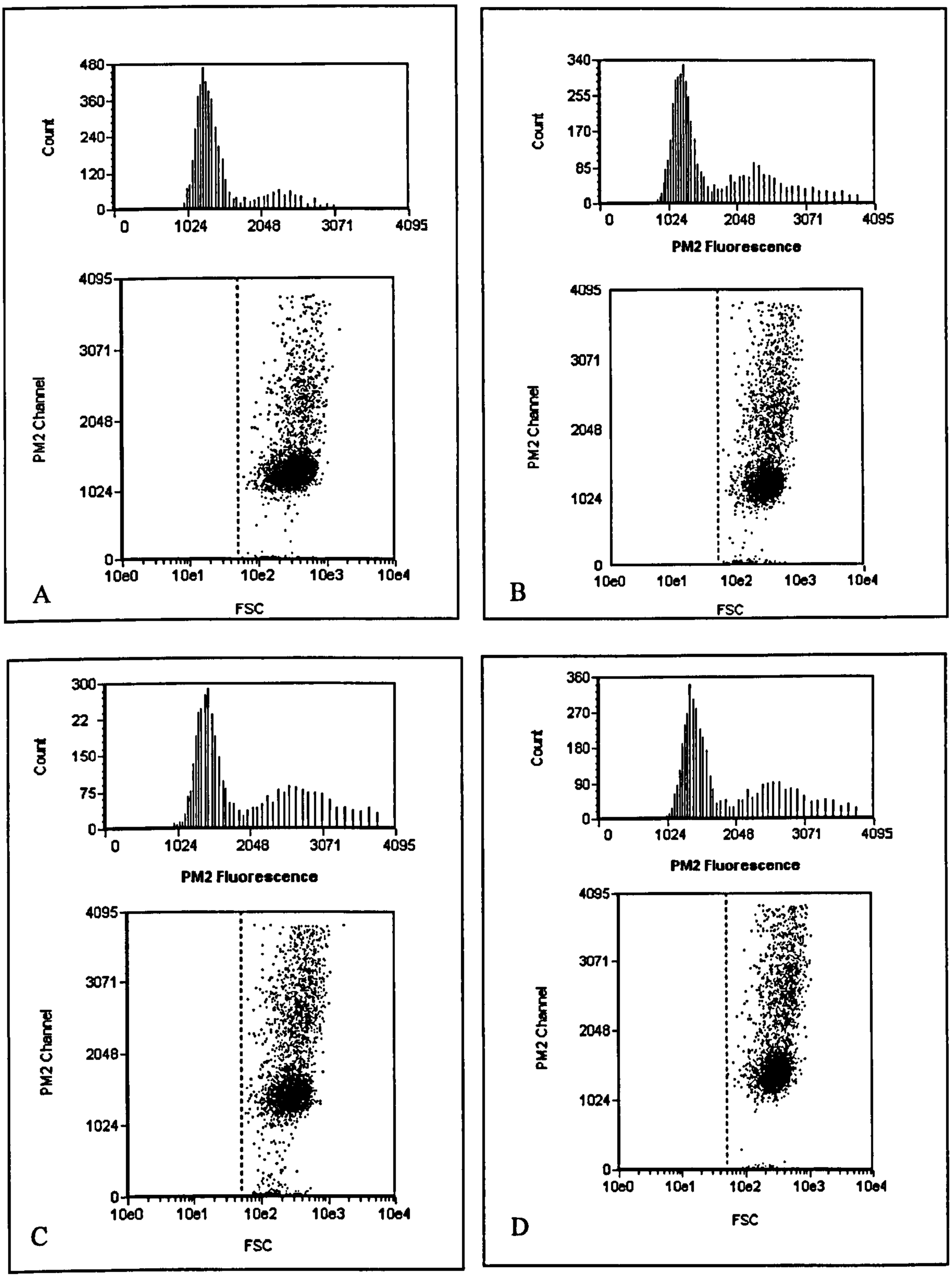


Figure 3-6: Representative images for cell cycle distribution analysis determined by FACS for cells cultured on a range of fibrin substrates (A)-3 (B)-10 and (C) 30 mg/ml) and a (D)-TCP control.

3.3.5.4 CD antigen characterisation of primary MSC cultured on fibrin gels

Substrate			CD antigen			
			Negative control	90	45	44
	TCP		0.4	70.9	2.9	36.8
	Fibrin (mg/ml)	3	0.4	68.9	2.7	28.1
		10	0.6	78.6	3.1	18.9
		30	1.7	85.6	6.3	46.1

Table 3-3: CD antigen characterisation for Primary MSC after a 7-day culture period on fibrin gels and TCP. 2 ml of bone marrow was cultured on fibrin gels (3, 10, and 30 mg/ml) and a TCP control for 7- days in osteogenic medium at 37 °C. At which point, cells were detached and the % of cells positive for three CD antigens (90, 45 and 44) determined using FACS. Data is presented as a representative of three independent experiments. 2000 events were acquired for each sample.

Immunofluorescent flow cytometry is a technique commonly used in the evaluation of cell surface marker expression on MSC. Growth on the various substrates appeared to have no major effects on CD antigen expression by MSC. As expected the cells were highly positive for CD90 on all substrates (68-85% positive), somewhat less positive for CD44 (18-46%) and largely negative for CD45 **(Table 3-3)**.

3.3.5.5 Change in morphology with fibrin concentration

Changing in morphology assessed by visual evaluation using phase-contrast microscopy has been previously used to demonstrate changes in morphology over time (Mets and Verdonk, 1981). Morphology of the cells on the different substrates was observed in 25-cm^2 culture flasks until the cells became confluent on approximately day 7.

By day 5 the cells were displaying distinct morphologies with an elongated more fibroblastic morphology as the stiffness of the underlying substrate increased. This trend was not observed on the TCP **(Figure 3-7)**.

Even when the cells were confluent on approximately day 7 the differences in the morphology between the cells cultured on the different fibrin gels and TCP was still apparent. Cells cultured on the fibrin gels acquired a fibroblastic appearance sooner than cells seeded on the TCP and in turn formed a confluent monolayer more quickly **(Figure 3-8)**. Furthermore, visual inspection revealed that the orientation of the cells on the substrates were different with cells on the fibrin aligning along the same axis compared to an irregular "cobblestone" arrangement on the tissue culture plastic.

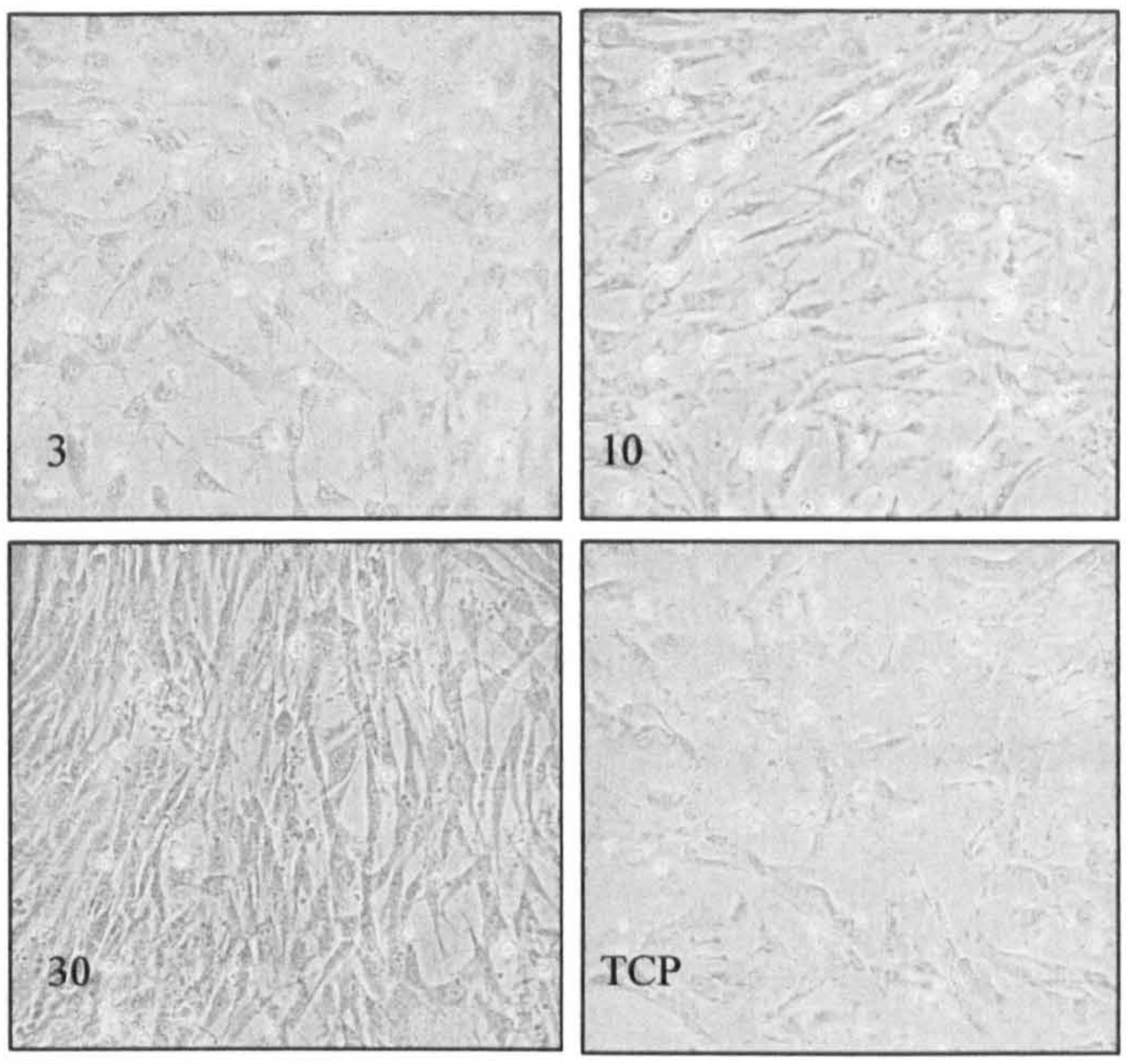


Figure 3-7: MSC morphology on fibrin gels and TCP on day 5. Phase contrast images to show the morphology of primary bone marrow derived MSC after 5 days in culture on a range of fibrin gels (3-30 mg/ml) and tissue culture plastic in growth medium (Mag x 10).

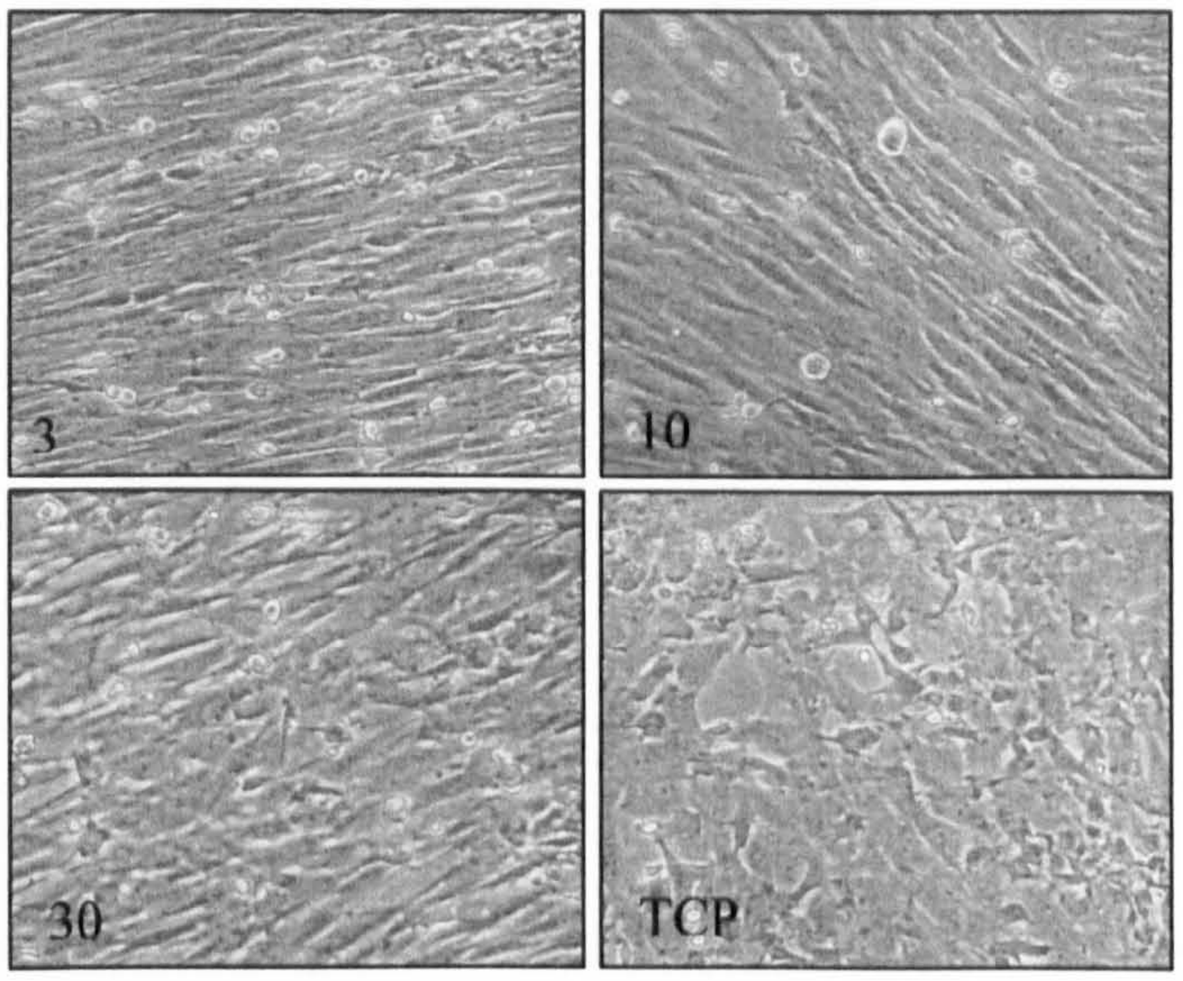


Figure 3-8: Morphology of confluent MSC on fibrin gels and TCP. Phase contrast images to show the morphology of primary bone marrow derived MSC after 10 days in culture on a range of fibrin gels (3-30 mg/ml) and tissue culture plastic in growth medium (Mag x10).

3.3.5.6 Comparison of relative averaged cell size with fibrin concentration

Changes in cell size by measuring forward-scatter flow cytometrically has been previously used to demonstrate differences in cell size with differing culture conditions (Sekiya et al., 2002). After culture on the fibrin gels for 7 days, analysis by forward light scattering showed that MSC cultured on the fibrin showed a trend, the size of the cell increased as the stiffness of the gel increased (**Figure 3-9**). As the stiffness increased the relative forward scatter increased from an average 369 ± 14 for 3 mg/ml to 400 ± 23 for the 10 mg/ml and then to 424 ± 6 for 30 mg/ml. The relative forward scatter for cells cultured on TCP was 368 ± 31.

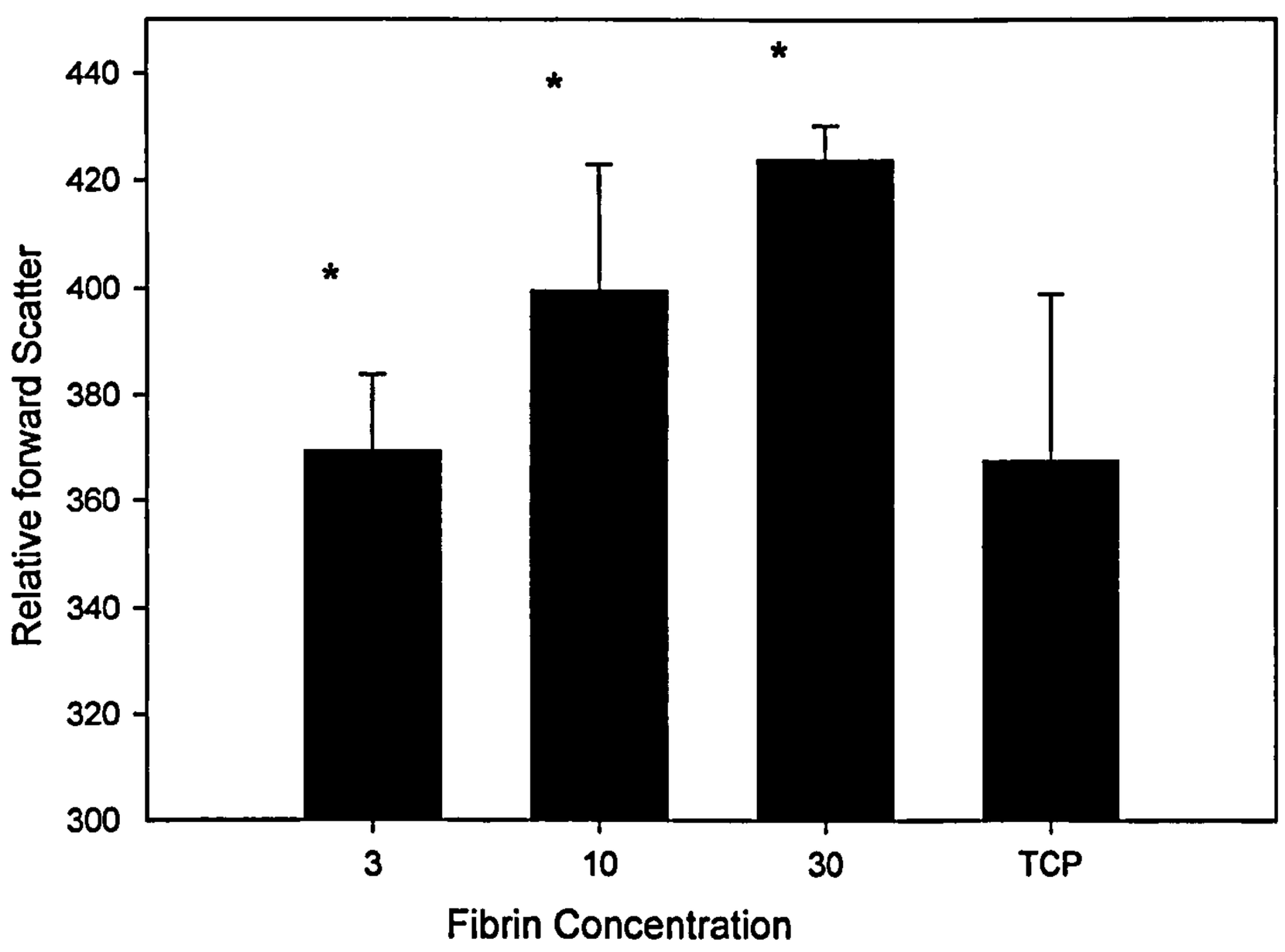


Figure 3-9: MSC relative cell size after culture for 7 days on fibrin gels and TCP. Primary bone marrow was cultured on the fibrin gels and TCP for 7 days. The average cell size of primary MSC was calculated based on an averaged forward scatter height after 1000 events were acquired. Data expressed as mean from three independent experiments ± SD (n = 3). * denotes significant difference from other fibrin densities.

3.3.5.7 Substrate type alters CFU-f efficiency

To determine the effect of an initial culture period on a fibrin substratum MSC were cultured on fibrin substrates (3, 10, 30 mg/ml) and TCP in standard growth medium for 7 days at which point, 1000 cells were transferred to uncoated 55-cm^2 petri dishes and cultured for a further 14 days in osteogenic growth medium. Staining with methylene blue and subsequent counting of the positively stained colonies shows the colony forming efficiency for each culture condition.

When compared to the TCP control, MSC that had been pre-cultured on the fibrin substratum showed a statistically significant increase in CFU-f efficiency. MSC pre-cultured on the softest substratum had over 100 % more total colonies compared to the TCP control. A decrease in colony number was seen as the substrate stiffness increased with an average of 106 ± 31 colonies on the 3 mg/ml, 83 ± 17 on the 10 mg/ml and 74 ± 7 colonies counted on the 30 mg/ml (**Figure 3-10**).

To determine the osteogenic potential of the MSC expanded on the fibrin, cells were cultured in osteogenic growth medium. The increase in colony number was also reflected by an increase in the number of colonies that were positive for ALP after pre-culture on the fibrin substrate compared to the standard TCP (**Figure 3-10**). ALP is a hydrolase enzyme commonly found in bone, amongst other tissues. Positive staining with napthol phosphate/ fast red is regularly used as a histochemical marker for the detection of osteoblasts. Cells that had initially been cultured on the 3 or 10 mg/ml fibrin substrate had nearly a 3-fold increase in the percentage of colonies that were stained ALP positive compared to the cells pre-cultured on the 30 mg/ml and the TCP (**Figure 3-12**) with an average of 50 ± 9 colonies on the 3 mg/ml, 39 ± 9 on the 10 mg/ml, 17 ± 1 colonies counted on the 30 mg/ml and only 8 ± 3 on the TCP (**Figure 3-9**).

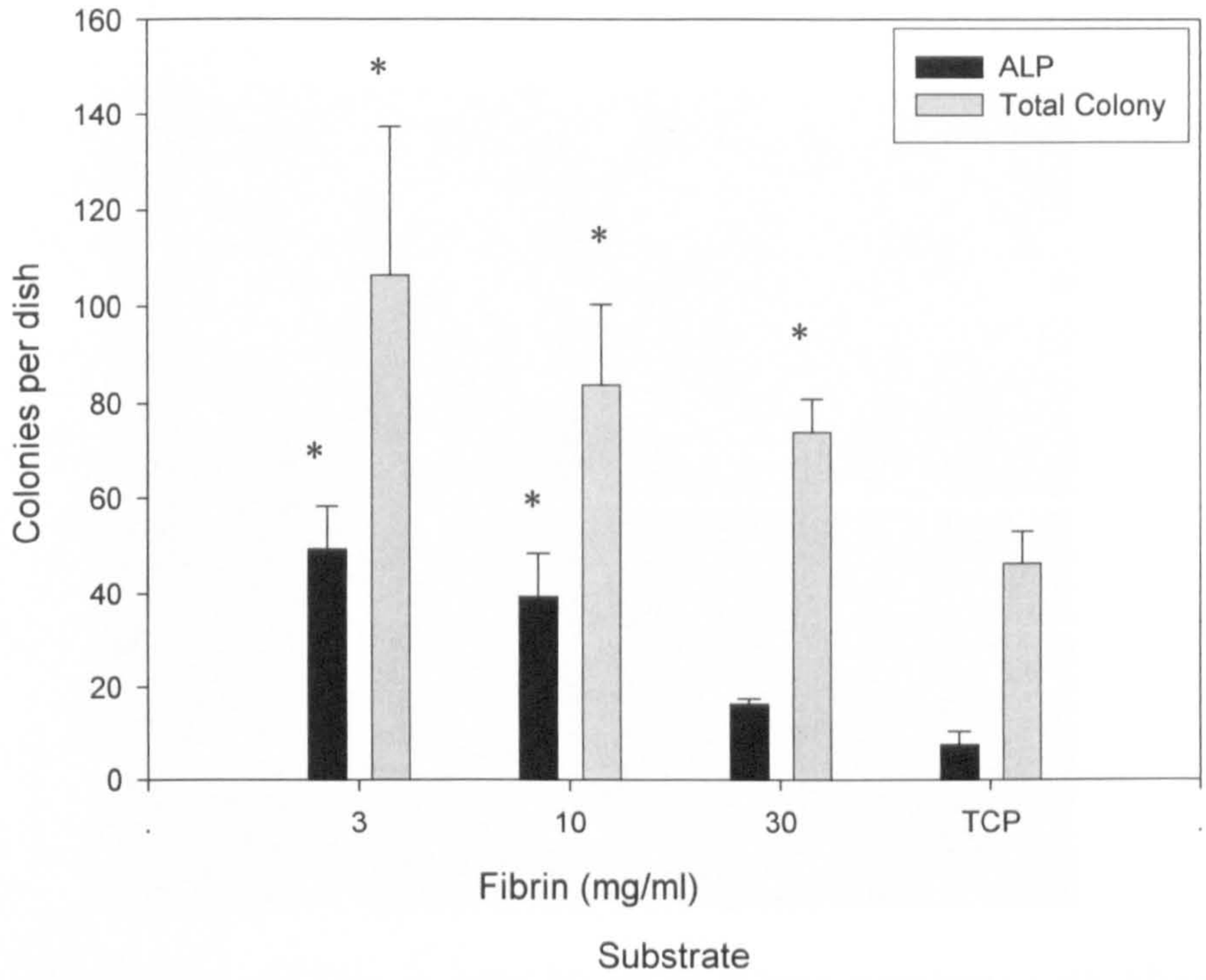


Figure 3-10: Distribution of CFU-f after P1 cell culture under osteogenic conditions. Primary MSC were cultured on fibrin gels (3, 10 and 30 mg/ml) and TCP controls for 7 days in standard growth medium. 1000 cells (P1) were then transferred to 55-cm^2 plates and cultured for a subsequent 14 days in osteogenic medium. After fixation, staining for ALP with naphthol phosphate/Fast red and for total colonies with methylene blue was performed. After each staining a digital image of each culture was acquired and the number and size of the colonies determined by image analysis. Data presented represents the mean colony number per 55-cm^2 plate after ALP and total colony staining ± SD (n = 4) representative of three independent experiments. * denotes significant difference from corresponding TCP control.

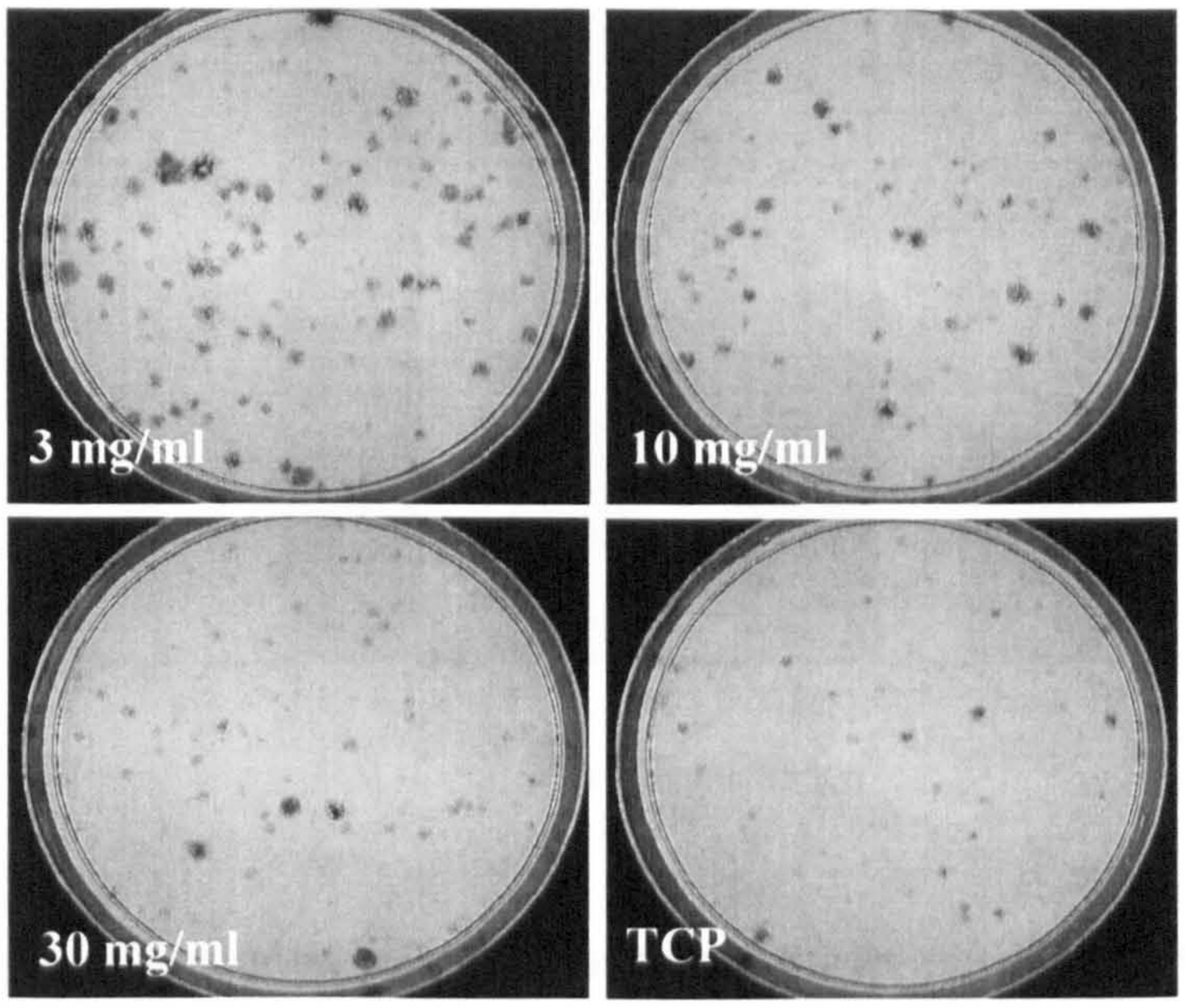


Figure 3-11: CFU-f cultures stained for total colony numbers with Methylene blue. Primary MSC were cultured on fibrin (3, 10 and 30 mg/ml and TCP control) for 7 days. 1000 cells were then transferred to 55-cm^2 petri dishes for CFU-f assay analysis and cultured for a further 14 days. This figure depicts a representative plate from each of the different fibrin concentration.

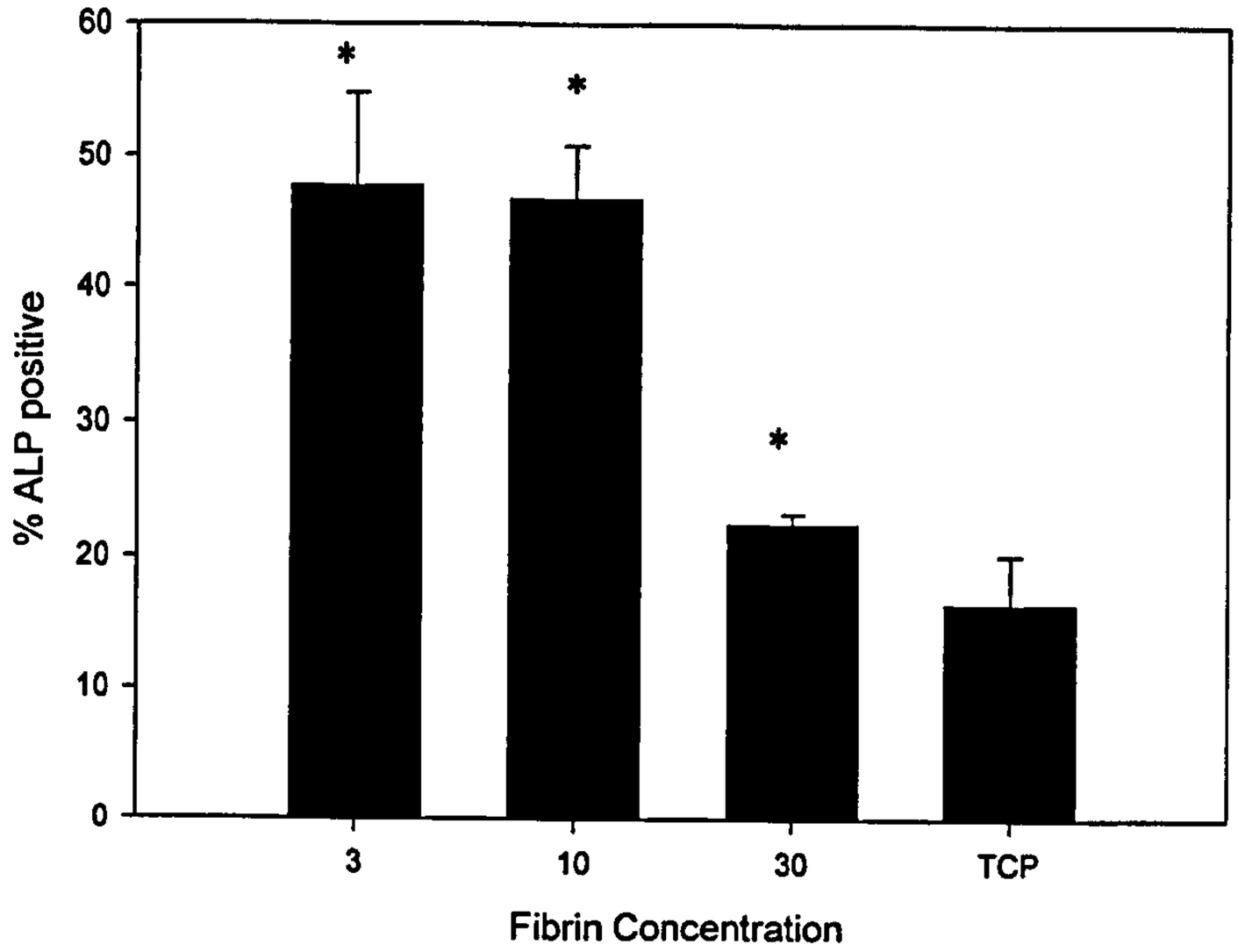


Figure 3-12: The Percentage of ALP positive colonies for each of the different fibrin concentrations and TCP control. * denotes significant difference from corresponding TCP control.

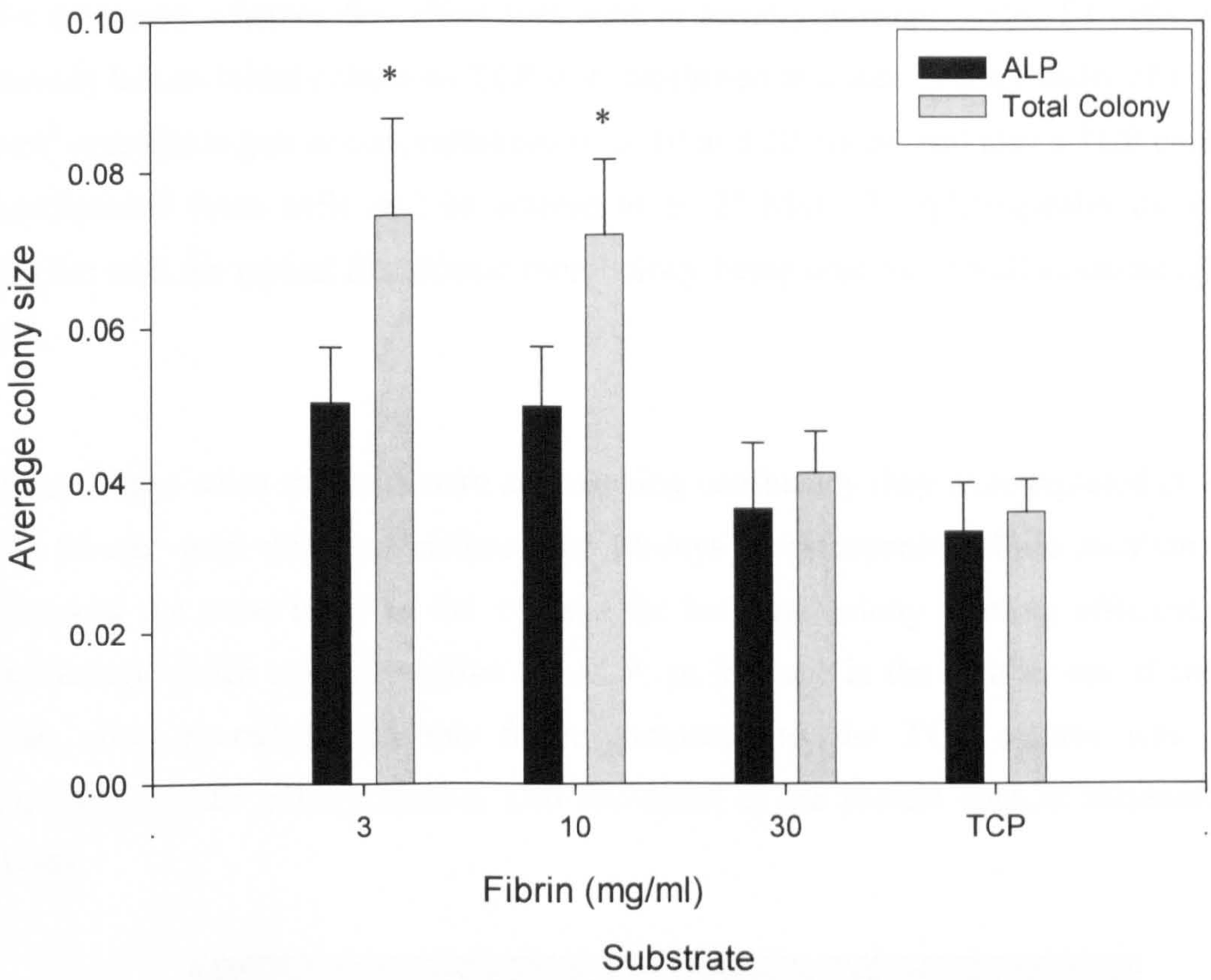


Figure 3-13: Average colony size per 55-cm^2 petri dish for both ALP positive cells and total colonies as determined by image analysis of the whole CFU-f culture. * denotes significant difference from corresponding TCP control.

To assess the proliferative capacity of the cells, average colony size was calculated for both the ALP positive colonies and for the total colonies. It was seen that total colony size was significantly larger on the fibrin substrates at both the 3 and 10 mg/ml concentrations when compared to the TCP control. There was no significant difference however between the average colony size which were stained positive for ALP, from each of the different substrates used (**Figure 3-13**). The average total colony size was always larger than the ALP positively stained colony regardless of pre-culture substrate type. This difference was larger for the CFU-f that had had an initial culture on the 3 and 10 mg/ml with the total colony size being approximately 48 % larger than the ALP colony size and compared to only an average 10 % increase after a pre-culture on the 30 mg/ml and TCP.

3.3.5.8 Effect of fibrin gels on expansion and differentiation of 2° MSC

To determine whether this effect was seen in serially passaged cells, P1 cells which had already had an initial culture on TCP were trpsinised and seeded at a density of 1 x 10^4 cells /cm^2 onto fibrin gels at concentrations of 3, 10 and 30 mg/ml and also a TCP control, from hereforward these cells will be refered to as 2° MSC. Morphologically the cells were similar with the typical fibroblastic morphology being observed on all substrates **(Figure 3-14).**

After 7 days when the cells were approaching confluency they were replated at 1000 cells per 55-cm^2 petri dish and cultured for 14-days in osteogenic culture medium. 2° MSC followed the same trend as the 1° cells for both the colony forming efficiency and the percentage which stained positive for ALP; an increase in the number the of colonies per dish after pre-culture on the fibrin compared to the TCP control was observed. Furthermore, the colony number also decreased as the protein content increased (**Figure 3-16**).

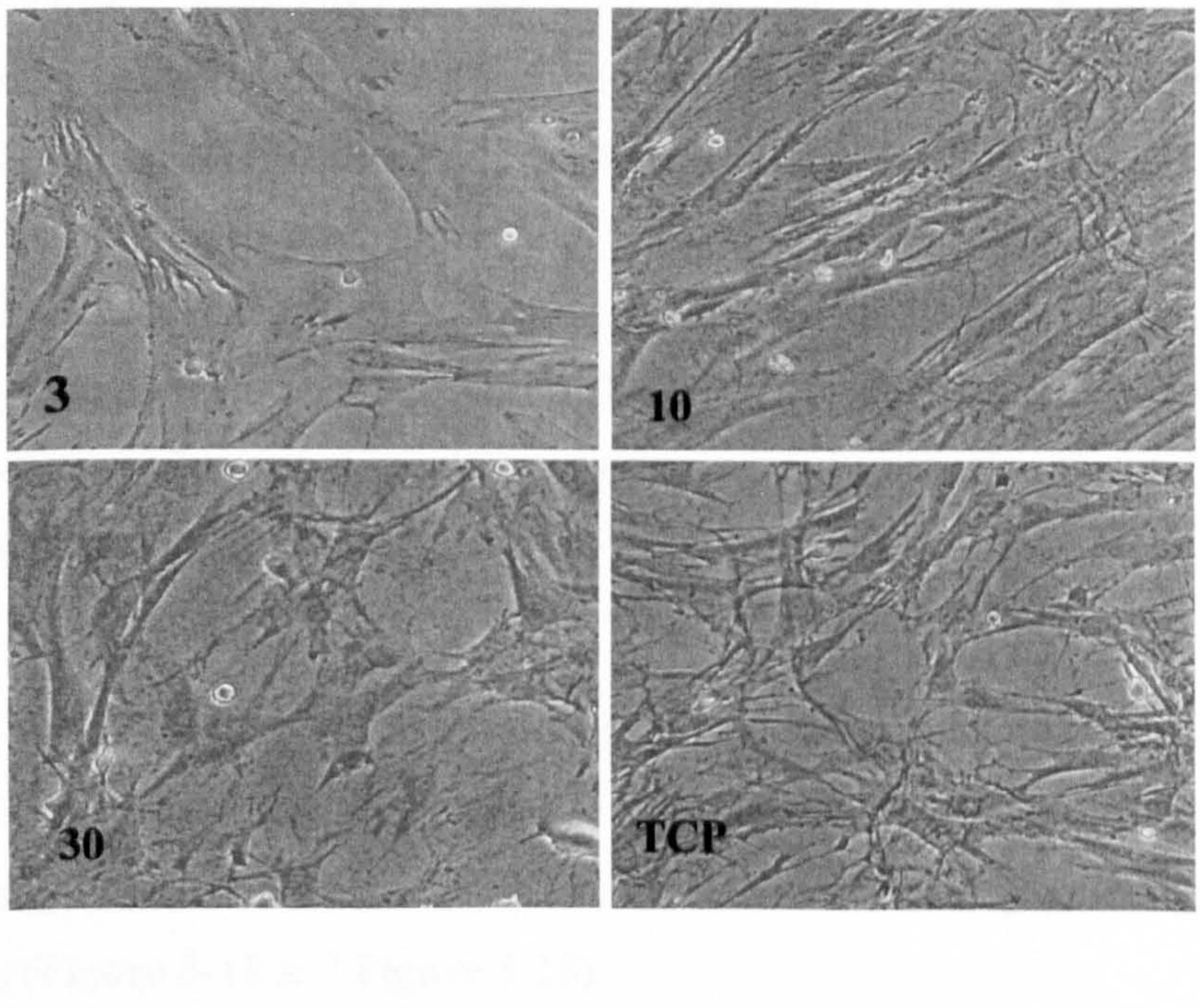


Figure 3-14: Morphology of secondary bone marrow derived MSC after 7 days in culture on a range of fibrin gels (3-30 mg/ml) and TCP. The MSC were initially cultured on TCP and then transferred to fibrin after 7 days (Mag x 10).

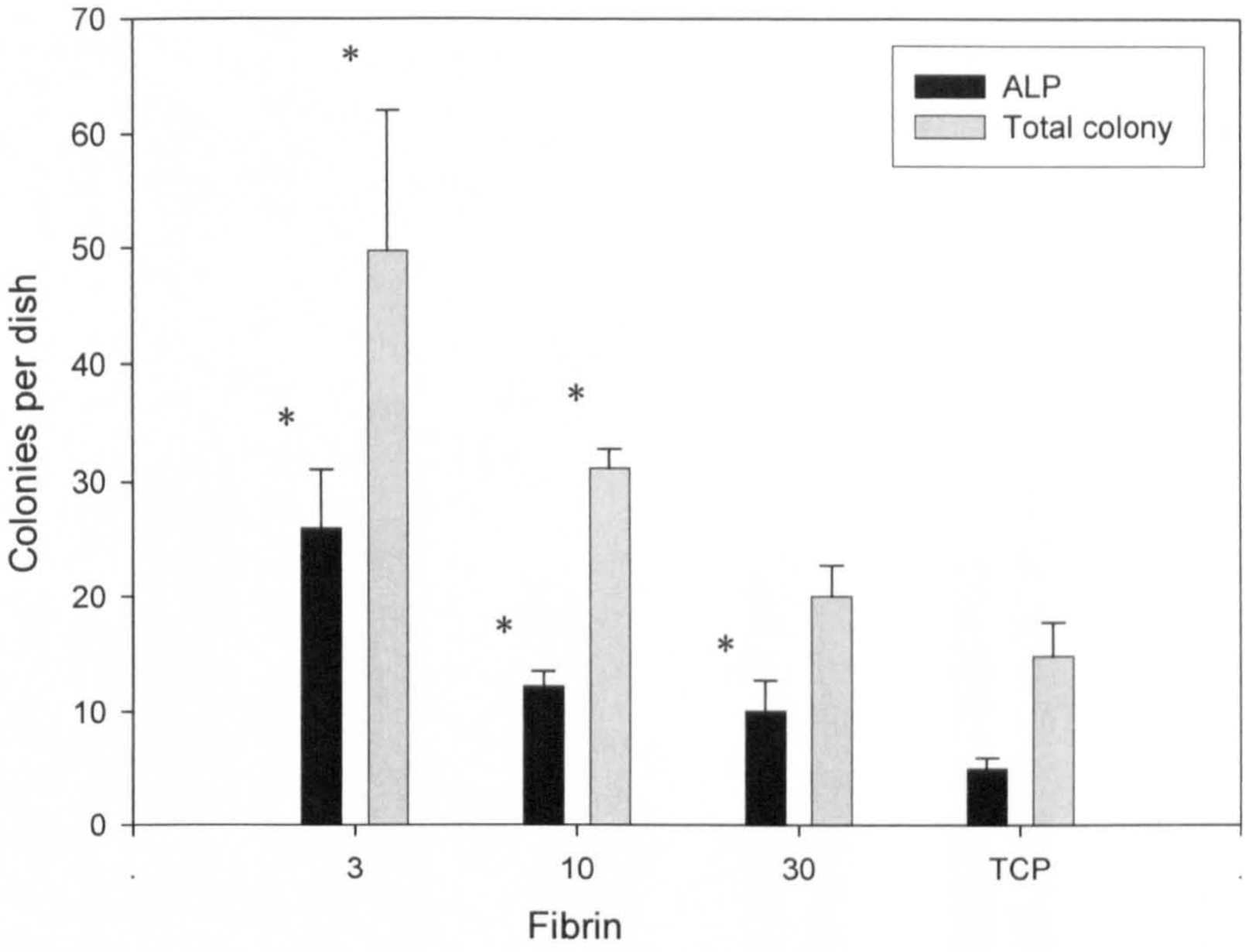


Figure 3-15: 2° MSC were cultured on fibrin gels and TCP for 7 days. Then, 1000 cells (2°) were transferred to 55-cm^2 plates and cultured for 14 days in osteogenic medium. After fixation, staining for ALP with naphthol phosphate/Fast red and for total colonies with methylene blue was performed. After each staining a digital image of each culture was acquired and the number and size of the colonies determined by image analysis. Data represents mean colony number per Petri dish after ALP and total colony staining ± SD (n = 4), representative of three independent experiments. * denotes significant difference from corresponding TCP control.

However, unlike the primary cells, no difference in average colony size was seen between the different groups and although the average size of the ALP stained colonies were smaller than the average total colony size the difference seen was not as great as that seen for the primary cells (**Figure 3-13** and **Figure 3-16**).

Similar to the primary cells, the percentage of colonies positive for ALP was highest for the cells pre-cultured on the fibrin gels compared to the TCP by an average of 14 % with all three of the fibrin substrates producing a higher percentage of ALP positive colonies compared to the TCP (**Figure 3-17**).

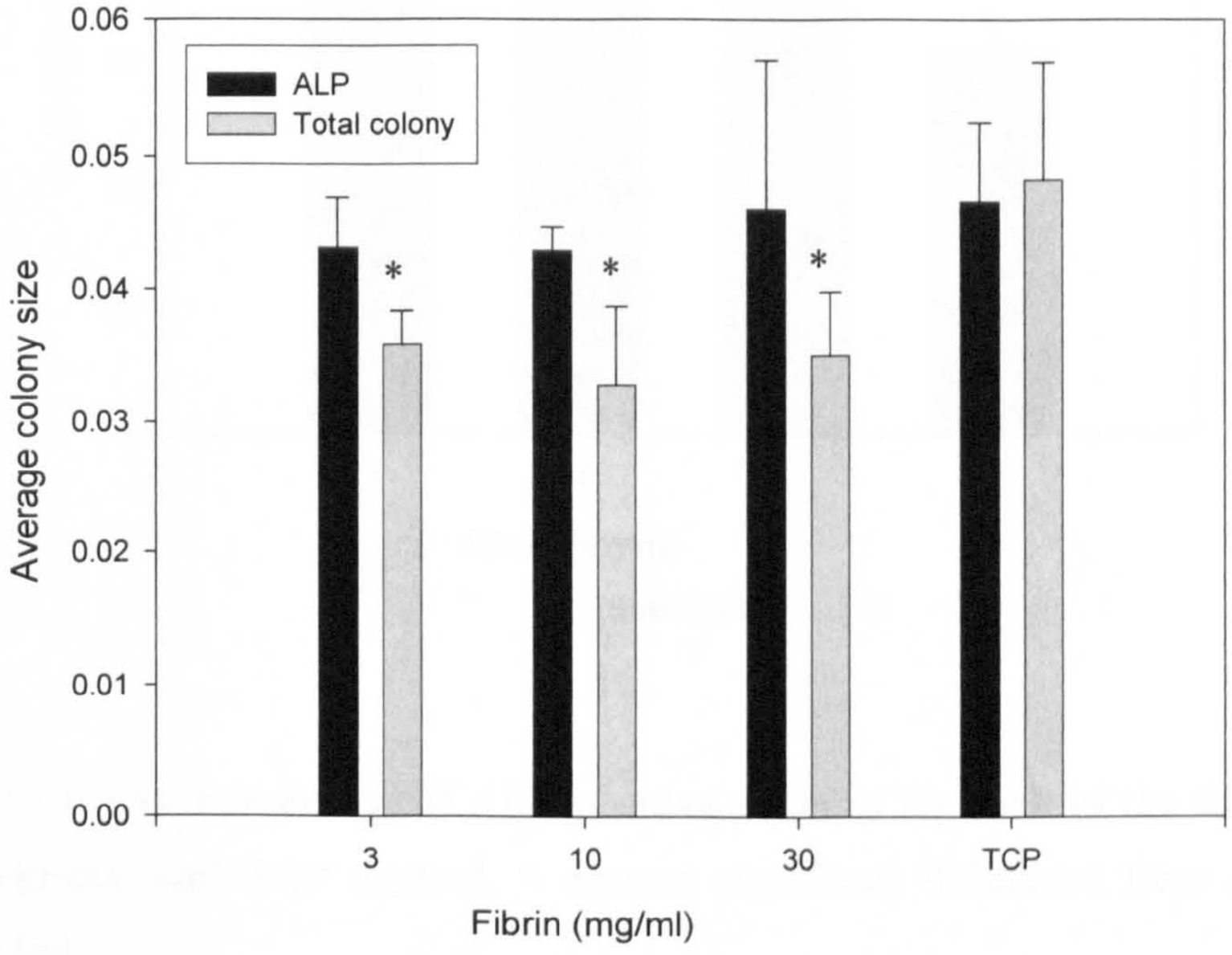


Figure 3-16: Average colony size for P2 MSC per 55-cm^2 petri dish for both ALP positive cells and total colonies as determined by image analysis of the whole CFU-f culture. * denotes significant difference from corresponding TCP control.

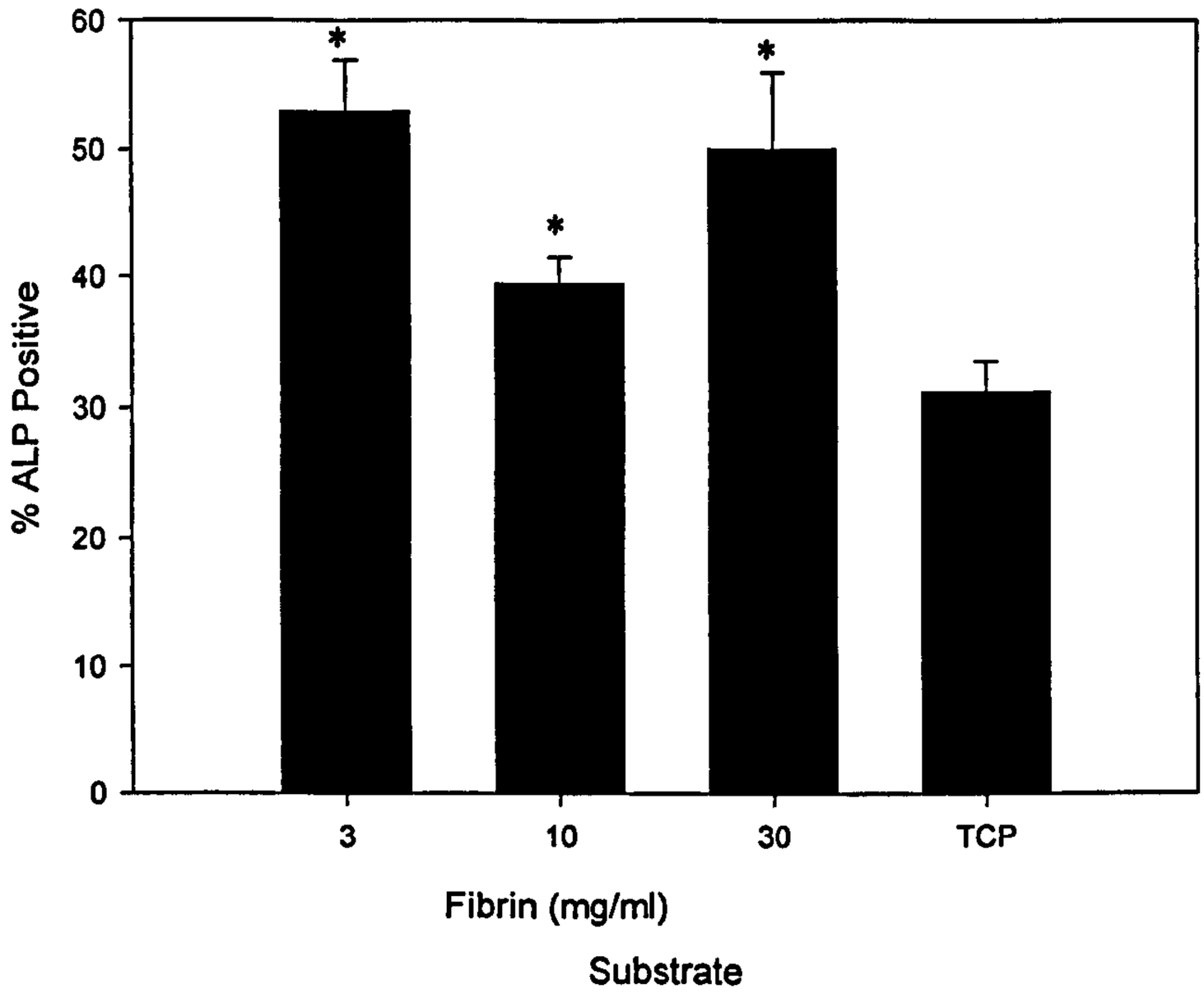


Figure 3-17: The Percentage of ALP positive colonies for each of the different fibrin concentrations and TCP control. * denotes significant difference from corresponding TCP control.

3.3.5.9 High density monolayer cultures

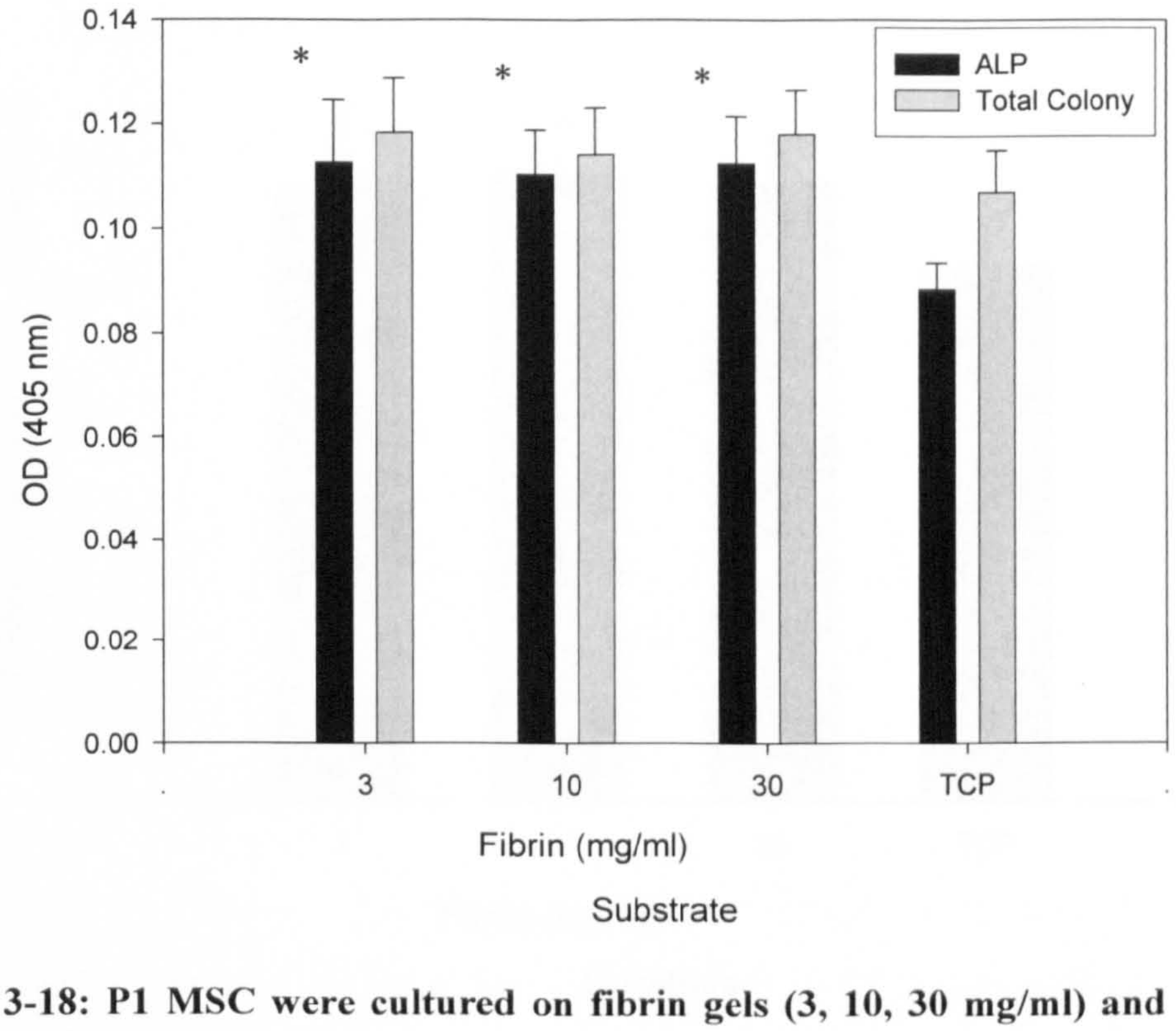


Figure 3-18: P1 MSC were cultured on fibrin gels (3, 10, 30 mg/ml) and TCP for 7 days, 1000 cells (P2) were subsequently cultured as high-density cultures. The cells were cultured under osteogenic conditions until they became confluent. After fixation, quantitive staining for ALP and total cell number was completed. The graph presented here depicts the ALP expression and total cell number for the different substratum ± SD (n = 6). * denotes statistically significant difference from the TCP control.

When cells were plated as a high-density culture, after pre-culture on the fibrin and TCP, the difference in the expression of ALP between the fibrin groups was diminished (**Figure 3-18**). However, a statistically significant difference was still seen in the ALP expression between pre-culture on the fibrin and TCP (**Figure 3-18**) but once this data was normalised to the number of cells present the ALP expression was not significantly different (**Figure 3-19**).

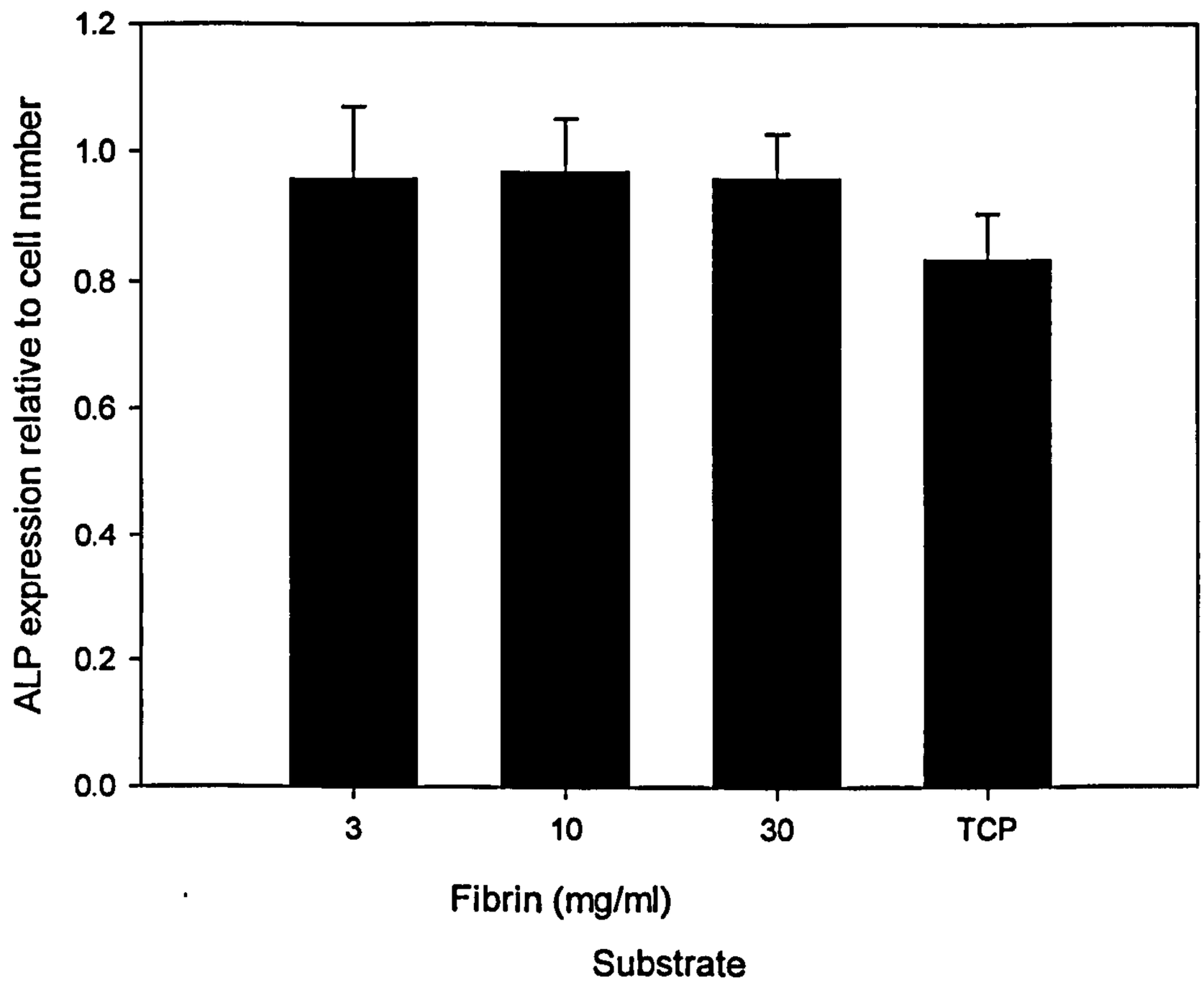


Figure 3-19: P1 MSC were cultured on fibrin gels and TCP for 7 days, 1000 cells (P2) were subsequently cultured as high-density cultures. The cells were cultured under osteogenic conditions until they became confluent. After fixation, quantitive staining for ALP and total cell number was completed. The graph presented here shows ALP expression relative to cell number ± SD (n = 6).

3.3.5.10 Differentiation of MSC after direct culture on fibrin gels

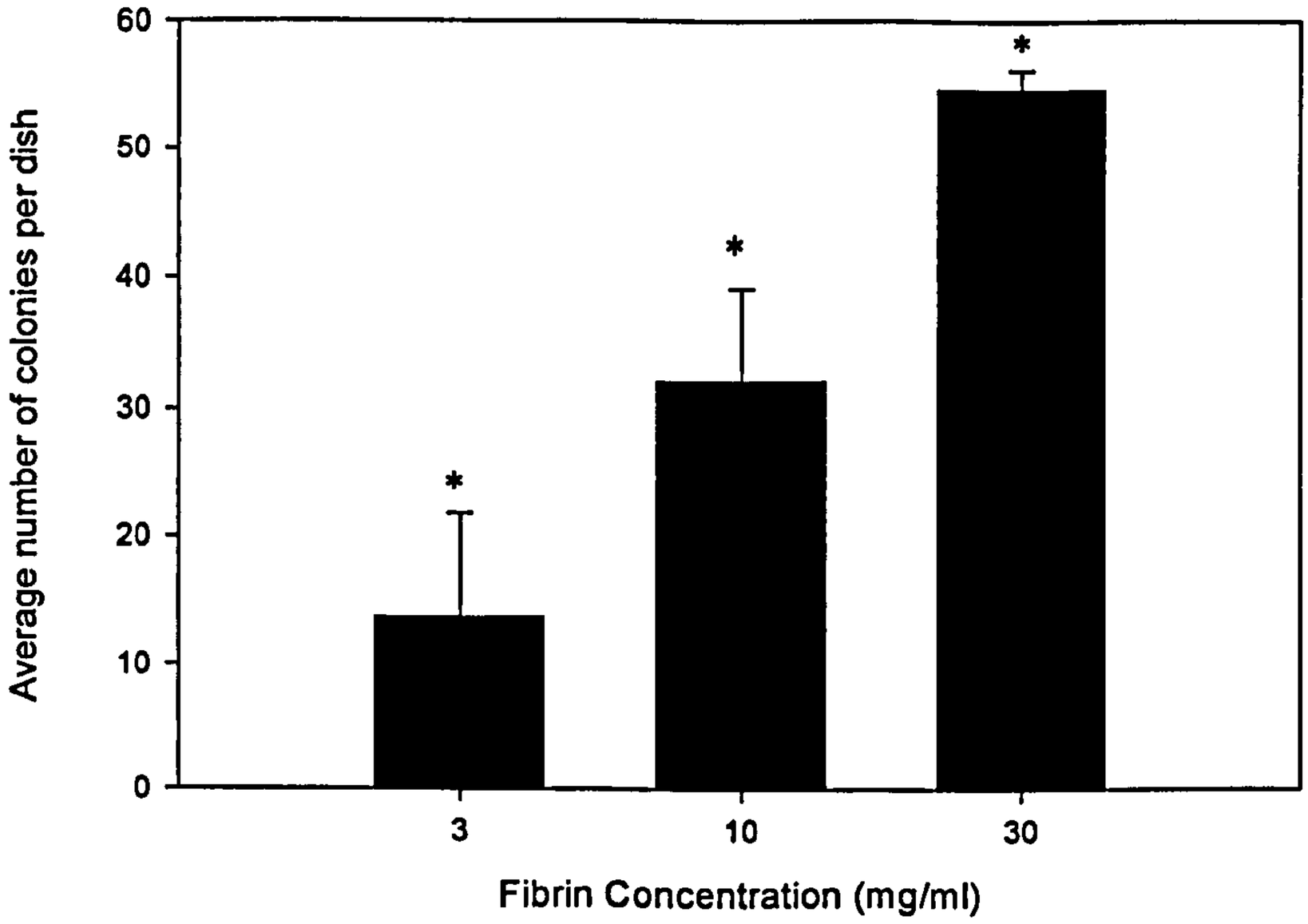


Figure 3-20: 500 µl of primary bone marrow was cultured in 55-cm^2 petri dishes as a CFU-f assay on a range of fibrin gels (3-30 mg/ml) in osteogenic culture medium. At 14 days the cultures were stopped and subsequently stained with napthol phosphate/fast blue for ALP expression. * denotes statistically significant difference from corresponding samples (n=4).

To determine if culturing MSC on fibrin gels had an effect on cell differentiation a CFU-f assay was completed in fibrin coated 55-cm^2 petri dishes. 500 µl of primary bone marrow cells were added to already fibrin coated 55-cm^2 plates and cultured for 14 days under osteogenic conditions. After fixation and staining with Napthol phosphate/Fast blue for ALP expression, the positively stained colonies were counted. **Figure 3-20** shows the number of positively colonies per plate, which increases as the stiffness on the gel increases from an average of 13.7 ± 8.1 for the 3 mg/ml to 32.0 ± 7.1 and 54.7 ± 1.5 for the 10 mg/ml and 30 mg/ml, respectively. However, due to the presence of the fibrin, quantification of the total number and size of the colonies was difficult to determine due to the absorption of

the stain into the fibrin gel. Quantification using the image analysis software was also not possible so counting was performed by directly counting the colonies. **Figure 3-21** shows representative images of the plates for each culture condition. The amount of differentiation could also be seen on an individual colony level. As the fibrin gel got stiffer the level of osteogenesis increased as observed by the intensity of fast blue staining. Also the level of adipogenesis was seen to be concentration dependant with more adipocytes on the softer substrates even under osteogenic conditions.

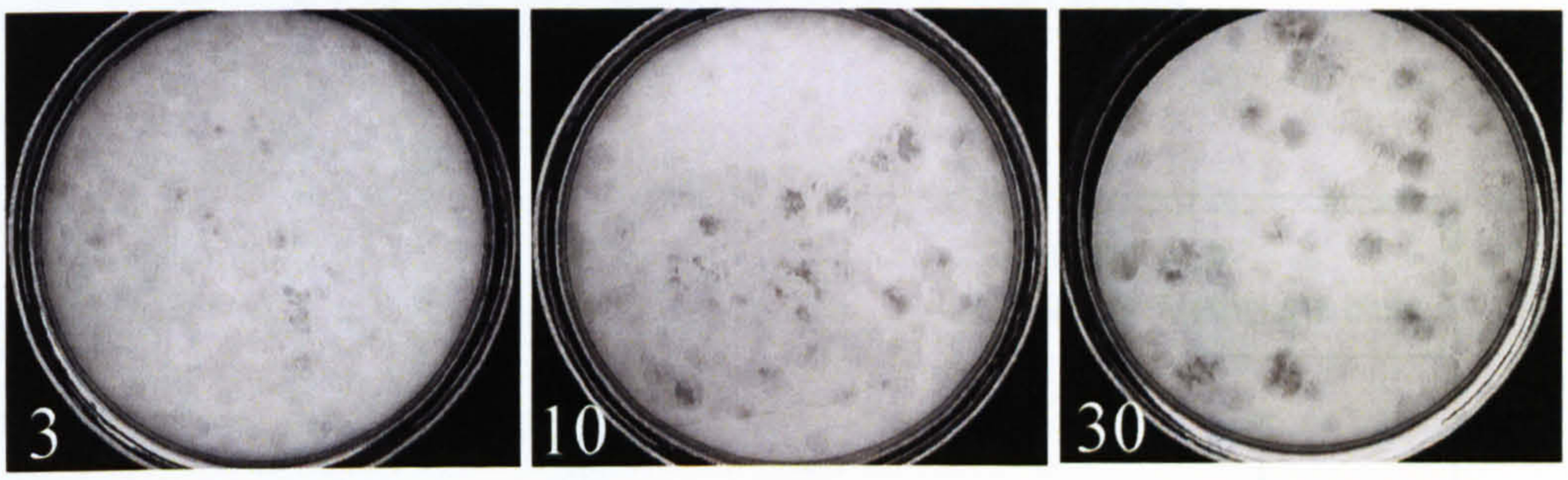


Figure 3-21: 500 µl of primary bone marrow was cultured in 55-cm^2 petri dishes as a CFU-f assay on a range of fibrin gels (3,10,30 mg/ml) in osteogenic culture medium. At 14 days the cultures were stopped and subsequently stained with Napthol phosphate/Fast blue for ALP expression. The images are representative of each substrate condition (N=3).

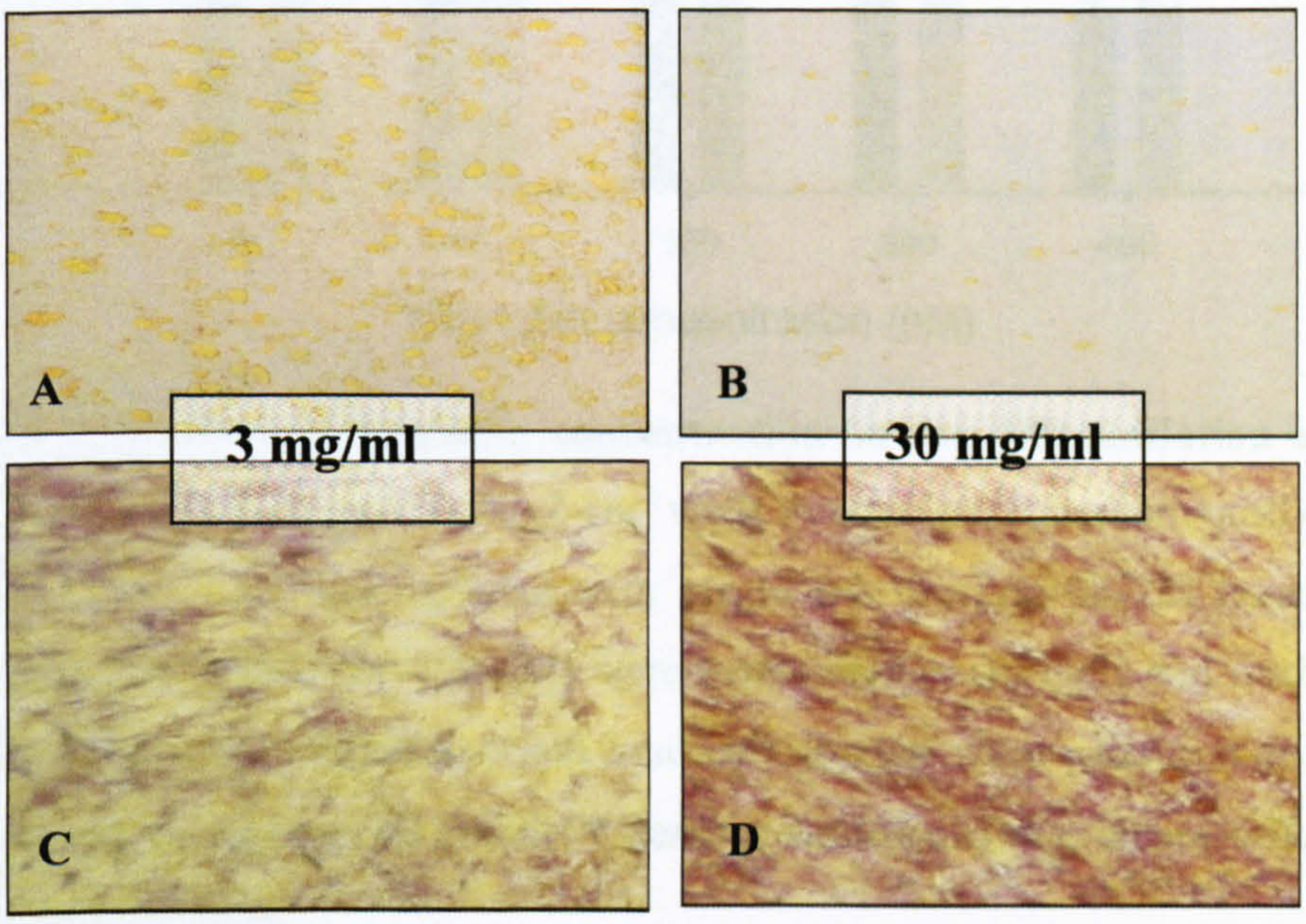


Figure 3-22: Bright field light microscopic images of CFU-f colonies. 500 µl of primary bone marrow was cultured in 55 cm^2 petri dishes as a CFU-f assay on a range of fibrin gels (3, 10 and 30 mg/ml) in osteogenic culture medium. At 14 days the cultures were stopped and subsequently stained. (A) and (B) show microscopic images of an individual colony stained for adipocytes using oil red O on 3 and 30 mg/ml respectively. In (C) and (D) the cells have been stained with Napthol phosphate/Fast blue for ALP expression (Mag x10).

3.3.6 The effects of thrombin concentration on ALP expression

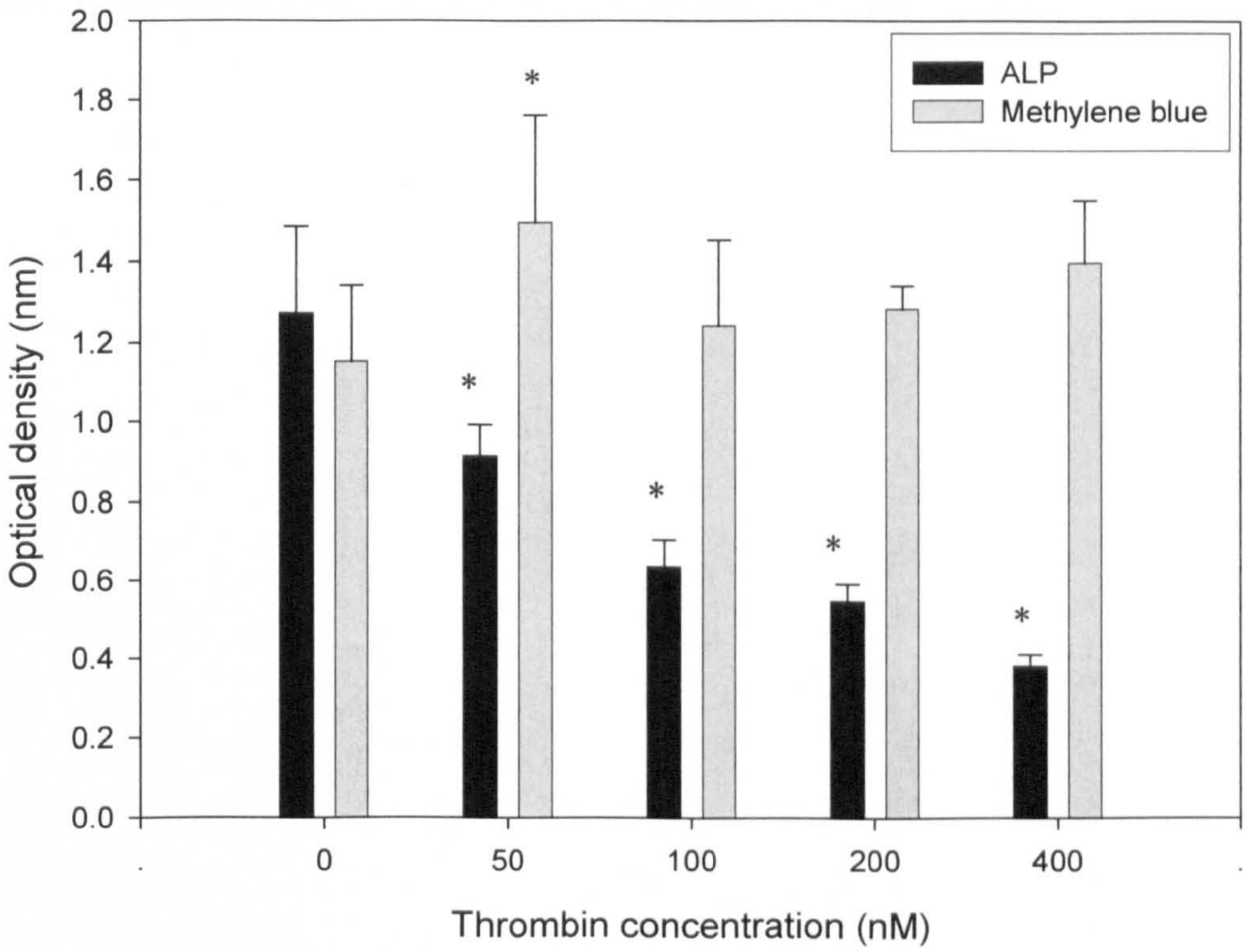


Figure 3-23: The effect of thrombin concentration on the differentiation and growth of MSC. 2 x 10^4 cells were cultured in 2-cm^2 wells in osteogenic medium until confluent at which point the medium was replaced with serum free medium for 24 hours. Different concentrations of thrombin (50-400 nM) were then added to the cells and further cultured for 48 hours before ALP expression and total cell number were measured quantitatively. Data is presented as mean ± SD (N=6). * denotes statistically significant difference from the corresponding control.

Although the amount of thrombin used in production of the fibrin substrates was kept constant, different concentrations of fibrinogen were used. This raises the question as to whether different concentrations of enzyme was present in the gels and whether the difference in fibrinogen concentration led to different concentrations of unreacted thrombin. As seen in **Figure 3-23** the amount of thrombin present has a significant effect on the expression of ALP with a dose dependant response seen. 100 nM of thrombin was

enough to approximately reduce the ALP expression by 50 %. Although thrombin had a significant effect on the differentiation of MSC, the presence of the protease had no effect on the growth of the MSC under serum free conditions (Figure 3-23).

3.4 Discussion

Fibrin, a natural biodegradable polymer, is being increasingly used in tissue engineering applications as a cheap, easily produced, biomimetic scaffold (Jockenhoevel et al., 2001). In the present study, we were able to demonstrate that fibrin gels can be produced with a range of elastic moduli by simply altering the fibrinogen concentration. Differences in the stiffness of the gels did not eaffect cellular attachment on two out of the three samples but differences in the rate of proliferation and viability levels were seen over an extended culture period. Also, the cell's morphology was typically more fibroblastic and orientated on the fibrin substrates when compared to the standard TCP substrate. After an initial selection and pre-culture on the fibrin gels, a concentration dependant increase in both the CFU-f efficiency and the percentage of cells capable of osteogenic differentiation was seen. Furthermore, a direct culture on the gels lead to a stiffness related change in osteogenic differentiation, with more osteogenic colonies as the substrate increased. The data presented here demonstrates that fibrin is a viable scaffold for the culture of MSC *in vitro* and can be used for the study of substrate stiffness on stem cell growth. In addition the data highlights the possibility that expanding MSC on a physiological substrate could lead to the maintenance of a more "stem cell" like phenotype in that the cells retain their clonogenic capacity and osteogenic potential as determined by the CFU-f assay.

In the presented study we used three different concentrations of fibrinogen (3, 10 and 30 mg/ml), whilst keeping the concentration of thrombin constant. We believed that these concentrations covered the stiffness range attainable from fibrin gels produced under these conditions as shown previously by Boewers et al, (2002). Although using this preparation technique offers only a limited range of stiffnesses compared to the artificial PDMS substrates, the stiffest of the gels (30 mg/ml) was over four fold stiffer compared to the softest gel (3 mg/ml), with the 10 mg/ml intermediate between the two. Previously, the elastic moduli of fibrin gels have been determined with investigators either calculating the Young's or shear modulus. In doing so, numerous techniques have been employed including rheometry (Ryan et al., 1999) and uniaxial tension (Sierra et al., 2002). For the purpose of this study we performed compression testing and then from the data calculated the tangent modulus (as there was no linear region on the stress/strain plot). Due to the wide range of techniques and conditions used in the literature, it is difficult to collate and

compare data concerning the elastic properties of fibrin and calls for the standardisation of a technique and type of modulus calculated, thereby enabling comparison between different research groups.

Here we have demonstrated that changing the fibrinogen concentration can simply alter the stiffness of the fibrin gels. However, it must be taken in to consideration that whilst the thrombin was kept constant, different concentrations of fibrinogen were used. Any results obtained possibly linking the behaviour of stem cells to substrate stiffness must take into consideration this difference in protein concentration. The change in protein concentration is a limitation often accounted whilst using natural substrates for this purpose and highlights the advantages of an artificial system where the surface chemistry can be clearly defined. However, the use of a natural substrate offers a number of advantages, including the offer of an autologous system that thereby eliminates any immunological concerns. The fibrin matrices also offer a physiological environment that is porous and allows a good nutrient flow under the cell that is not typically present in *in vitro* culture using the standard TCP.

Suitability of a potential biomaterial for the production of scaffolds is often assessed by cell attachment and subsequent growth over time in culture (El-Amin et al., 2003). The quantative cell attachment data seen in **Figure 3-5** was flawed, due to the fact that it was very difficult to count the cells accurately. The assay was attempted in small 2-cm^2 wells causing the gels (especially the softest gel) to have a meniscus and therefore the cells were prone to attaching in the middle of the well. This explains the large standard deviations observed, as the fields of view for cells to be counted were always selected at random. It was also very difficult to accurately count all the cells in a field of view due to the differences in the focusing caused by the topography of the gels. When trying to count the cells using the automated system after staining with dapi there was evidence that the cells had not been fixed properly or that the gel was degraded whilst staining. Alternative methods should be sort to accurately quantify cell attachment. The data obtained suggested that attachment was higher on the fibrin substrates compared to the TCP control and could explain the difference seen in proliferation on day 2 (**Figure 3-3**) as more cells could have attached initially. Attachment assays were completed using 2° cells and therefore does not

give a clear picture as to whether the fibrin gels had an effect in the selection process of the MSC from the bone marrow cells. Further attachment assays are needed to determine if more cells are actually being selected from the bone marrow on any of the different gels.

In the measure of cell proliferation, conventional reduction based assays employed for the measurement must be used with caution if the substrate is porous. Here we found that even the comparison between different fibrin gels were inaccurate as eluted cell culture medium can interfere with the sensitivity of the colorimetric indicator. Due to this it was decided that the cells would be detached and counted directly.

In this study it was observed that MSC are able to adhere, spread and proliferate when seeded upon the fibrin gel matrices regardless of the elastic moduli. Primary MSC cultured on different concentrations of fibrin and TCP showed a difference in proliferation over an 11-day period, with the cells growing quickest on the softer substrates (3 and 10 mg/ml). This effect was seen to deplete over-time, as no statistically significant difference was seen after day 9 and therefore suggests that cells on the stiffer substrates have a longer lag-phase. Other reports have shown that when cells are seeded within fibrin gels a concentration higher than 18 mg/ml is too dense to allow spreading and subsequent growth (Bensaid et al., 2003) but for monolayer purposes this doesn't seem to be the case and is a consideration for 3D culture only. Previous studies have also shown that the expandability of MSC cannot be predicted from the initial growth rates of low passage cells (DiGirolamo et al., 1999) and would be a consideration if the effects of culture on fibrin gels was to be assessed over a longer-time frame and highlights that long-term expandability cannot be estimated from an initial culture period.

For the re-plating assays (CFU-f and HDC), the cells were first cultured for 7 days (until approximately 80 % confluent) in fibrin-coated flasks before passaging on to TCP 55 cm^2 plates. As the cells proliferated at different rates on the fibrin gels, cell cycle analysis was performed to determine whether at the time of re-plating the cells were in the same stage of the cell-cycle and to determine if this difference in growth could be having an effect on the CFU-f potential at this chosen time-point. It was found that at this point there were no variation in the number of cells in each stage of the cell cycle and although the cells

cultured on the TCP had more cells in G_0/G_1 and fewer in G_2M the result was not significant.

Culture on the different fibrin gels substrates had a slight effect on cell viability but only at the start (day 2) and the end (day 11) of the culture period. Viability levels determined by fluorescent dead/live staining revealed that cell viability was high regardless of differences in the elastic properties of the substrates. Cells cultured on the 30 mg/ml were however seen to have statistically significant lower viability levels at the beginning of the culture period which could be related to the fact that the fibrin gel structure was too dense as previously described (Bensaid et al., 2003). Although no difference was seen in the initial attachment of cells on this substrate (compared to the other fibrin groups) it is possible that the cells needed longer to spread before being able to proliferate. This could also explain the longer lag phase observed in the growth curve. The % viability of cells cultured on the 3 and 10 mg/ml were seen to decrease slightly (statistically significant) at the 11-day time point compared to the other two substrates and is probably due to the cells becoming confluent quicker on these substrates and the initiation of contact inhibition.

The viability data presented is a measure of the percentage of viable cells in a given population and does not take into consideration viable cells that have recently initiated the apoptosis pathway. To rectify this, apoptosis levels were also determined. During apoptosis phosphatidylserine is translocated from the cytoplasmic face of the plasma membrane to the cell surface. Annexin V has a strong affinity for phosphatidylserine and is therefore used in the detection of apoptosis. Although not significant, apoptosis levels were higher as the stiffness of the gels increased and could again be indicative to the fact that the cells do not like the increase in protein concentration, making the matrix too dense. A assay to measure cell size over time on the different substrates would have been able to show if the cells were unable to sufficiently spread and possibly link too dense a matrix with the difference in apoptosis levels seen.

The initial attachment rate of 1° MSC (**Figure 3-3**) shows that an increased number of cells attached to the softer fibrin substrates compared to the 30 mg/ml and the TCP. To determine if there was a difference in the cell type that was adhering from the bone marrow

stromal cells, the expression of a number of cell surface markers was assessed. Immunofluorescent flow cytometry is a technique commonly used in the evaluation of cell surface markers expression on MSC. The technique was use to ascertain whether the fibrin substrates were promoting the attachment of a different population of primary cells from the bone marrow. The markers used (CD90, CD44, CD45) are typical markers used in the assessment of MSC. No significant difference in the range of markers was seen **Table 3-3** and therefore eliminates the fact that the different concentrations of fibrin attracted different cell populations at this time point. This assay was completed on day 7 as MSC are typically selected by their adherence to the substrate and an initial culture period is necessary to eliminate the unbound cell fraction that is usually lost after the first medium change. It would have been interesting to have had completed this assay at different time points and to look for 'more stem cell like' markers to see if culture on a more physiological substrate does lead to the maintenance of a more 'stem cell' like phenotype.

There was a distinct difference in the morphology of the cells after 5 days in culture, not only between the fibrin and the TCP but also between the different fibrin concentrations Cells on the fibrin gels took on a more elongated, fibroblastic morphology as the stiffness increased. This trend was not seen on the TCP with the cells appearing "cobblestone" like. Furthermore, cells cultured on the fibrin gels when confluent were observed to be oriented with one another, aligning along the same axis. Again, cells on the TCP were observed to have the typical 'cobble-stone' like appearance. Sun et al., (2006) reported that cell orientation is essential for retaining normal tissue properties and functions and cells *in vivo* are in an organised, spatially arranged pattern. Research has been undertaken looking in to the effects of spatial patterning on MSC proliferation and differentiation (Sekiya et al., 2002), but limited work has been completed looking at cell orientation and a possible link with MSC behaviour. It has also been suggested that the correct organisation leads to the favourable attachment and strength of adhesion mediated through focal contacts and is highlighted by the fact that an irregular orientation is observed in pathophysiology (Weiss et al., 1991). It is possible that the fibrin gel causes the cells to align and orientate better due to a more physiological adhesion or because the cells are receiving a better nutrient flow, due to the porous nature of the material. Not only were differences seen in the morphology of the cells but also the size of the cell was seen to be different. Cell size has

previously been linked to cellular function including progression through the cell cycle (Ostuni et al., 2000). It is well accepted that an increase in cell size is linked to senescence of the cell and thereby suggests that smaller cells are "younger" then the larger ones (Baxter et al., 2004). The FSC data presented in **Figure 3-9** showed that MSC cultured on the softer concentrations of fibrin were significantly smaller than those cultured on the stiffer substrates.

MSC isolated from bone marrow are easily expanded in culture and are able to differentiate into a number of cell lineages including osteoblasts, adipocytes and chondrocytes (Pittenger et al., 1999). Although MSC are easily isolated, low numbers *in vivo* means a need for exponential expansion and enrichment *in vitro* before use therapeutically (Chen et al., 2004). Loss of multipotency as the cells are expanded is also seen in culture (DiGirolamo et al., 1999), a characteristic that may have important implications where large numbers of cells are needed. The use of the CFU-f as an assay to determine the number of MSC present in a cell preparation has been well characterised (Dobson et al., 1999a). The assumption that each colony arises from a single cell allows for the number of colonies to be correlated to the number of MSC in the original sample. The CFU-f results presented here demonstrate that an initial seven-day plating onto fibrin affects the growth, subsequent CFU-f efficiency and differentiation potential of both primary (**Figure 3-10**) and early passage MSC (**Figure 3-15**). It was observed that not only an increase in CFU-f efficiency, but also the number of colonies that stained positive for ALP was higher after pre-culture on the softer substrates. This data suggests that pre-culturing or initially sorting for cells on the softer substrates maintains a more 'stem-cell' like phenotype in that the cells retain their clonogenic capability and an increased osteogenic differentiation potential. A study investigating MSC expansion and maintenance of phenotype has implicated the basement membrane ECM as a critical factor in the proliferative life span and maintenance of differential potential of stem cells in culture and highlights the importance of a native, soft substratum for maintaining a more 'stem cell' like phenotype (Matsubara et al., 2004).

One explanation for the occurrence of a cell population that maintained "stem cell" like properties on the lower concentrations of fibrin is that there is a selection for smaller, more spindle shaped cell or that the softer substrates maintain a population with a smaller cell

size. It has previously been demonstrated by Mets and Verdonk, (1981) that colonies isolated from a single pre-cursor give rise to cells with heterogeneous morphologies. The work has been extended further by classifying the cells into two categories, recycling stem cells (RS cells)-a population of small, rapidly self renewing cells and a mature, larger cell that is somewhat granular (mMSC) (Mets and Verdonk, 1981, Colter et al., 2000). It was further hypothesised that MSC that proliferate the quickest, retain their multipotent phenotype for longer. This hypothesis was exemplified by the fact that, RS cells demonstrate a higher level of multipotency, with the observation made that larger cells are slower to replicate and have less differentiation potential (Colter et al., 2000, Colter et al., 2001). The FSC data presented here supports this theory with the cells on the softer substrates being smaller, proliferating quicker and also maintaining differentiation potential. Differentiation potential of the cell has also been linked to cell shape. It is suggested that a further division of the RS cells can be made into RS-1A, RS-1B and RS-1C as determined by the morphology of the cell in culture. Differences in the number of cells in each of the sub-populations is determined by culture conditions including initial seeding density and culture time with enrichment for the RS-1A and RS-1C cells giving rise to an increased number of adipocytes and chondrocytes respectively. It is possible that the differences in morphology witnessed here are also having an effect on the differentiation potential of the MSC.

Although the average FSC for cells cultured on the TCP was similar to those cultured on 3 mg/ml, the range of the cell size was seen to be a lot larger than for the cells cultured on the fibrin gels. It is therefore possible that the MSC are becoming morphologically homogeneous quicker on the fibrin gels compared to the TCP. Typically MSC are only morphologically homogeneous after several passages (DiGirolamo et al., 1999).

The difference in colony number for cells cultured on the different substrates could however be due to an artefact of cell clumping. As only a small number of cells were being re-plated, an automated counting system was used to eliminate the degree of human error involved and it is therefore possible that there was a degree of cell clumping caused when the cells were removed from the fibrin substrates. Contrary to this, determination of the

percentage of ALP positive colonies for the whole population demonstrated on average a 2-fold increase on the 3 and 10 mg/ml compared to the 30 mg/ml and TCP (**Figure 3-12**).

The difference in the number of colonies positively stained for ALP could be linked to the average colony size with larger colonies being more pre-disposed to an osteogenic phenotype. Work completed by Stein and Lian, (1993) illustrates that a high density of cells is necessary for osteoblastic differentiation. In the work presented here, it was found that colonies were larger on the 3 and 10 mg/ml substrates (**Figure 3-13**). Possibly with the cells on the softer gels being able to proliferate quicker with no space restrictions.

The difference in colony number seen between p1 (**Figure 3-10**) and p2 cells (**Figure 3-15**) was expected as colony number is known to decrease with passage number (DiGirolamo et al., 1999). The reproducibility of this result using passage 2 cells was however poor compared to the passage 1 cells and was possibly due to the low number of colonies obtained. Further investigation would be needed with the possibility of using more cells for the re-plating assay to yield a higher number of colonies for further analysis.

The results for the CFU-f assay were reflected in a high-density culture between the fibrin groups and TCP, yet the effect seen between the different fibrin groups was diminished. However, when relative to cell number, there was no significant difference in ALP expression between any of the culture substrates. This supports previous work which has established that MSC expansion on TCP with the maintenance of multipotent differentiation potential is preferential at low seeding densities (Sekiya et al., 2002). But is this practical? You would need 200 T75's to yield the cell number necessary for therapeutic applications (Chen et al., 2004) and demonstrates the need for a compromise between convenience and reliability!

The data obtained from the CFU-f of cells grown directly on the fibrin matrices showed that the number of positively stained colonies increased as the stiffness of the substrate increased (**Figure 3-20** and **Figure 3-21**). However, due to the presence on the fibrin gel, analysis of the plates could not be completed using the standard method as described in chapter two. Instead colonies were directly counted. Additional to an increase in colony

number, using bright light microscopy to look at individual colonies, it was observed that a more intense staining for ALP was seen on the stiffer substrates (30 mg/ml) when compared to the softer substrate (3 mg/ml) (**Figure 3-22**). Interestingly, even under osteogenic growth conditions there were more adipocytes on the softer substrates suggesting that adipogenesis is favoured on more flexible substratum. This data also highlights the fact that each colony is not homogeneous even though the cell population has arisen from one cell. It has been proven in the past that cells from the same clone under identical osteogenic conditions can be conditioned to behave differently (DiGirolamo et al., 1999). This data supports the findings that the cell is already pre-conditioned and that soluble factors are not enough alone to always cause differentiation. Stem cell fate is controlled by a number of conditions including temperature (Stolzing and Scutt, 2006), cell density (Sekiya et al., 2002) and ECM components (Matsubara et al., 2004) and is best exemplified by looking at the conditions for the induction of cartilage from MSC where a micro-mass conditions mimic *in vivo* cartilage formation (Pittenger et al., 1999).

It has previously been shown that thrombin stimulates proliferation whilst inhibiting differentiation in osteoblasts (Abraham and MacKie, 1999) but to the best of my knowledge the effect of thrombin on MSC proliferation and differentiation has not yet been investigated. Although the amount of thrombin added to cause gelation was kept constant at 1 unit/ml the percentage of thrombin in the system would be different due to the differences in fibrinogen concentrations and for these reasons we decided to investigate the effects of thrombin on MSC under osteogenic conditions. We showed that as the concentration of thrombin increased the expression of ALP decreased (**Figure 3-23**) but for the concentrations presented here, it is unlikely that this is having an effect with the system.

3.5 Conclusions

- Fibrin gels can be produced with varying stiffnesses
- Fibrin supports MSC monolayer culture without any need for further modifications
- MSC proliferate quicker on softer gels
- On fibrin gels viability and apoptosis levels are consistent with those seen for MSC in culture
- Morphologically the cells appear more elongated and fibroblastic on the fibrin substrates
- Cell size increases with fibrin gel concentration
- CFU-f efficiency is enhanced by a pre-culture on fibrin gels. A concentration-related response is observed with a decrease in colony number as the stiffness increases.
- Osteogenic potential is maintained after a pre-culture on the fibrin gels. Again, a concentration-related response is observed with a decrease in ALP positive colonies as the stiffness increases
- Differentiation to the osteogenic lineage is enhanced on the stiffer substrates when the MSC are cultured on the gels
- Furthermore, adipogenesis is favoured on the softer gels
- Thrombin inhibits the expression of ALP in a dose respondent manner

CHAPTER FOUR: The use of PDMS as a variable-stiffness substrate

4.1 Introduction

Most *in vitro* research is carried out on glass or plastic surfaces though most cells *in vivo* will be attached to a much softer material. With an increasing use of biomaterials in clinical applications including scaffolds for tissue engineering (Kim et al., 2000), medical devices (Haid et al., 1988), and biosensors (Pantoja et al., 2004) a greater understanding of the effects of substrate stiffness on cell behaviour is needed. Substrate stiffness has already been shown to influence a number of cellular processes including locomotion (Pelham and Wang, 1997), cell shape (McBeath et al., 2004) and proliferation (Wang et al., 2000).

The use of artificial flexible substrata in cell culture is not a new idea and started nearly 25 years ago when Harris et al, (Harris et al., 1980) developed a technique to use silicone rubber as a culture substratum, eliminating problems encountered by using protein substrates and their unpredictability. Although a limited number of substrates are currently being used including polyvinyl alcohol (Cho et al., 2005), glyoxyl agarose (Hohn et al., 1995) and polyacrylamide (Pelham and Wang, 1997), PDMS, most widely used silicon-based organic polymer, offers a number of advantages. Its good optical clarity, low permeability to water and its thermal stability all contribute to making it an ideal biomaterial but it is its easily controllable mechanical properties that make it highly desirable to study the effects of substrate stiffness on cell behaviour. By simply altering the ratio of siloxane oligomers and crosslinkers possible moduli of more than 1 MPa are attainable, a moduli nearly 10 times that of the well characterised polyacrylamide gel system and similar to moduli reported for human tissues (Wong, 2004).

Even though there are reports of cells adhering to an unmodified silicone substrata (Harris et al., 1980), cell adhesion is poor due to the hydrophobic nature of the material. Most other investigators have relied on non-covalent methods to enhance cell adhesion *i.e.* physisorption of fibronectin (Gray et al., 2003), however, recent studies found adsorption of matrix proteins to PDMS to be an inefficient method for enhancing cell attachment (Cunningham et al., 2002). Other methods are also being used and have been well described including surface oxidation using plasma treatments (Prichard et al., 2006), gelatine-glutaraldehyde cross-linking (Ai et al., 2002), and layer-by-layer polyelectrolyte coatings (Ai et al., 2003). However, none of these techniques are ideal for the study of

mechanical properties of the substrate on cell behaviour as researchers have encountered problems including coating instability, coatings being too thick, and inability to support long-term cell culture.

For this study we have used plasma polymerisation, a gaseous coating technique to coat nano-thin polymeric films on to surfaces to enhance cell adhesion. It is becoming a very attractive technique in the field of biomaterial as the plasma deposition is possible onto almost any solid material, with little or no pre-treatment (Daw et al., 1998). A number of studies have shown that plasma deposited thin films can be used to both improve (France et al., 1998) and eliminate (Shen et al., 2003, Bretagnol et al., 2006) cell adhesion and growth, depending on the coating chemistry and the monomer used. The majority of plasma-based surface modifications used at present for PDMS involve plasma treatments using oxygen to create hydrophilic SiO_x groups at the surface or radicals that will enable the subsequent grafting of pre-formed polymers at the surface (Hsiue et al., 1998). Previous work has shown that surfaces coated with plasma polymerised acrylic acid supports high levels of cell attachment, with studies suggesting that the carboxylic acid group has more significance in cell attachment compared to the hydroxyl or carbonyl functionalities (Daw et al., 1998).

We developed and characterised the use of PDMS as an artificial substrate to study the effects of substrate stiffness on stem cell growth. Mechanical testing was completed to ensure that a range of moduli were attainable by altering the oligomer to crosslinker ratio. Different coatings were investigated to enhance cell attachment and the subsequent growth of the cells before the system was analysed using a number of biological assays including cell proliferation, viability and cell cycle analysis. Surface science techniques were employed to define the substrate system and ensure that all the variables were considered. The effects of substrate stiffness on MSC growth and differentiation were then investigated.

4.2 Materials and Methods

Refer to chapter two.

4.3 Results

4.3.1 Artificial substratum preparation

PDMS is available commercially as SYLGARD 184, a two-part kit consisting of 'curing agent' (crosslinker) and 'base' (oligomer) solutions. The manufacturers recommend a 1:10 w/w ratio for normal use but to create PDMS substrates with a range of elastic moduli different curing agent to base ratios were used (1:10-1:50). After 72 hours curing time, at room temperature, mechanical testing was completed to determine the Young's moduli of the different polymers.

4.3.2 Mechanical testing

4.3.2.1 Bulk Young's modulus

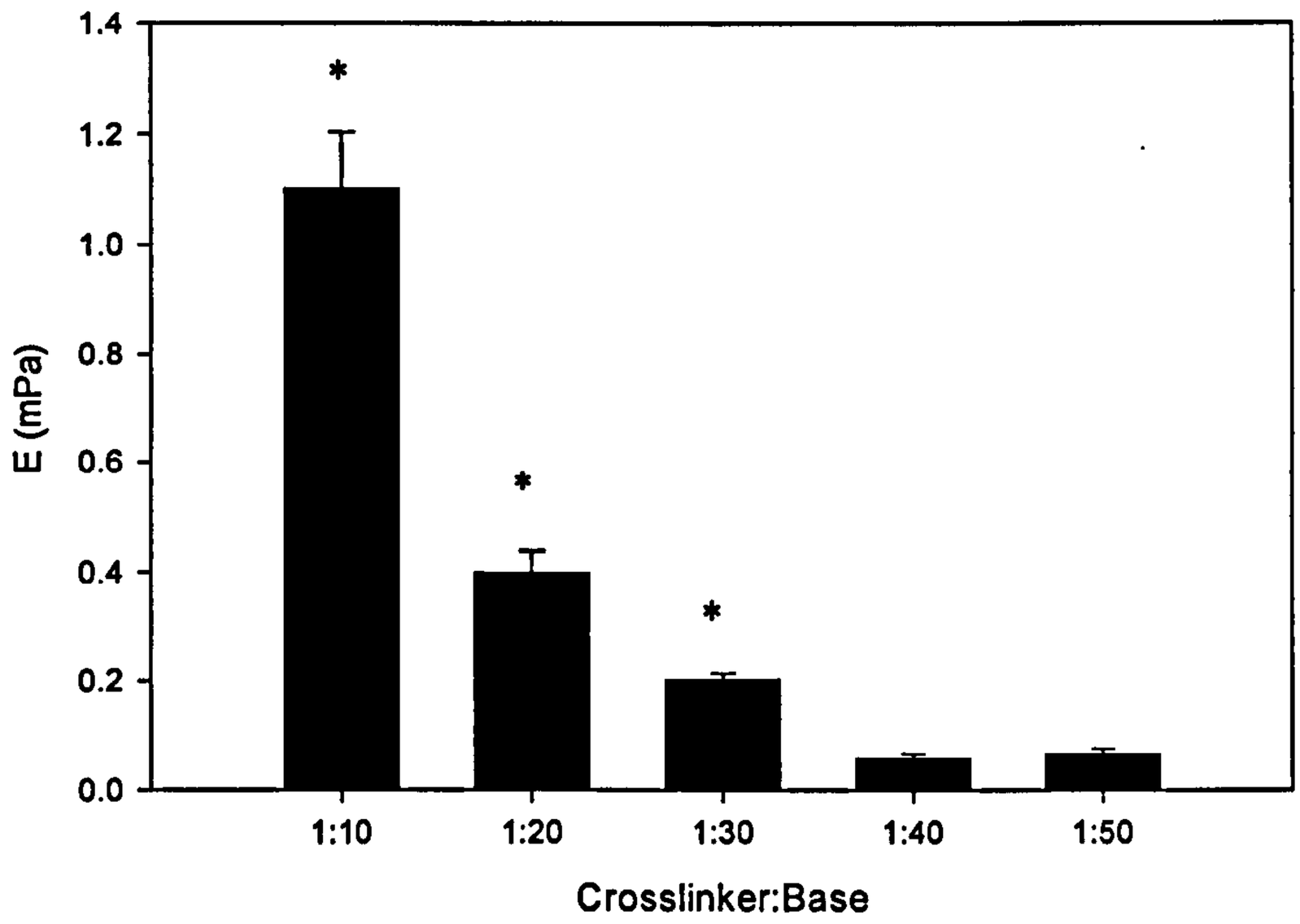


Figure 4-1: Elastic moduli for PDMS substrata as determined by tensile testing. The graph shows the Young's moduli (E) for a range of crosslinker to base ratios (1:10-1:50). Data shown are the mean of 5 samples ± SD. * denotes significant from next sample.

Tensile testing of the substrates using a tensile tester (Bose Electroforce 3200) showed that as the crosslinker to oligomer ratio increased from 1:10 to 1:50 the Young's modulus of the bulk material decreased from 1.1 MPa (± 0.1 MPa) to 66 kPa (± 9.08 KPa). A ratio below

1:10 did not increase the Young's modulus of the bulk material (data not shown) and substrates produced with a ratio higher than 1:50 could not be tested, as they were too difficult to handle due to incomplete polymerisation. A significant difference was seen between each of the samples except for the two softest substrates (1:40 and 1:50) (**Figure 4-1**).

From the initial tensile testing data it was decided that three ratios would be further characterised for possible cell culture substratum namely the 1:10, 1:30 and 1:50. These ratios represent the full range of attainable moduli and have significantly different Young's moduli.

4.3.3 PDMS as a cell culture substratum

For PDMS to be used as a biomaterial cells must be able to adhere, spread and proliferate when cultured upon it. Both cell adhesion and spreading are essential for subsequent growth and are therefore useful determinants to evaluate proposed tissue engineered surfaces. Cell proliferation rates also enable the sustainability of the cells on the substratum to be assessed and therefore gives further validation of the suitability of the material as a cell culture substratum. Tissue culture plastic was used as a positive control as it is the standard cell culture substrate and readily available.

4.3.3.1 Cell attachment

MSC were able to adhere within 3 hours to all samples of the uncoated PDMS regardless of crosslinker to base concentration and in both serum rich and serum free medium. However, in serum rich medium this initial attachment was seen to be approximately 2.5 fold lower when compared to the standard TCP. At the 24 hours time point, although the number of cell attached had increased by an average 23 % and 30 % for the 1:10 and 1:50 respectively there was no change in the number of attached cells for the 1:30. Although an increase was seen there was still an approximate 2.5 fold decrease when compared to the standard TCP.

A completely different pattern was seen in the serum free medium. After three hours the number of attached cells on the PDMS was seen to be nearly 4 fold greater compared to those in the serum rich medium. An average of 28 % greater cells were also seen on the PDMS compared to the TCP. More cells were also attached to the TCP in serum free

conditions compared to serum rich medium. However, this pattern of initial attachment had diminished by the 24-hour time point. In the serum free conditions less cells were seen on all of the substrates with an average reduction of 44 % on the PDMS substrates compared with the 3-hour time point. Regardless of this reduction, there were still more cells attached to the PDMS in serum free conditions compared to the serum rich, an effect not seen on the TCP. No significant differences were seen between the different PDMS substrates for any given condition or time point, except for the 1:30 in serum rich conditions at 24 hours.

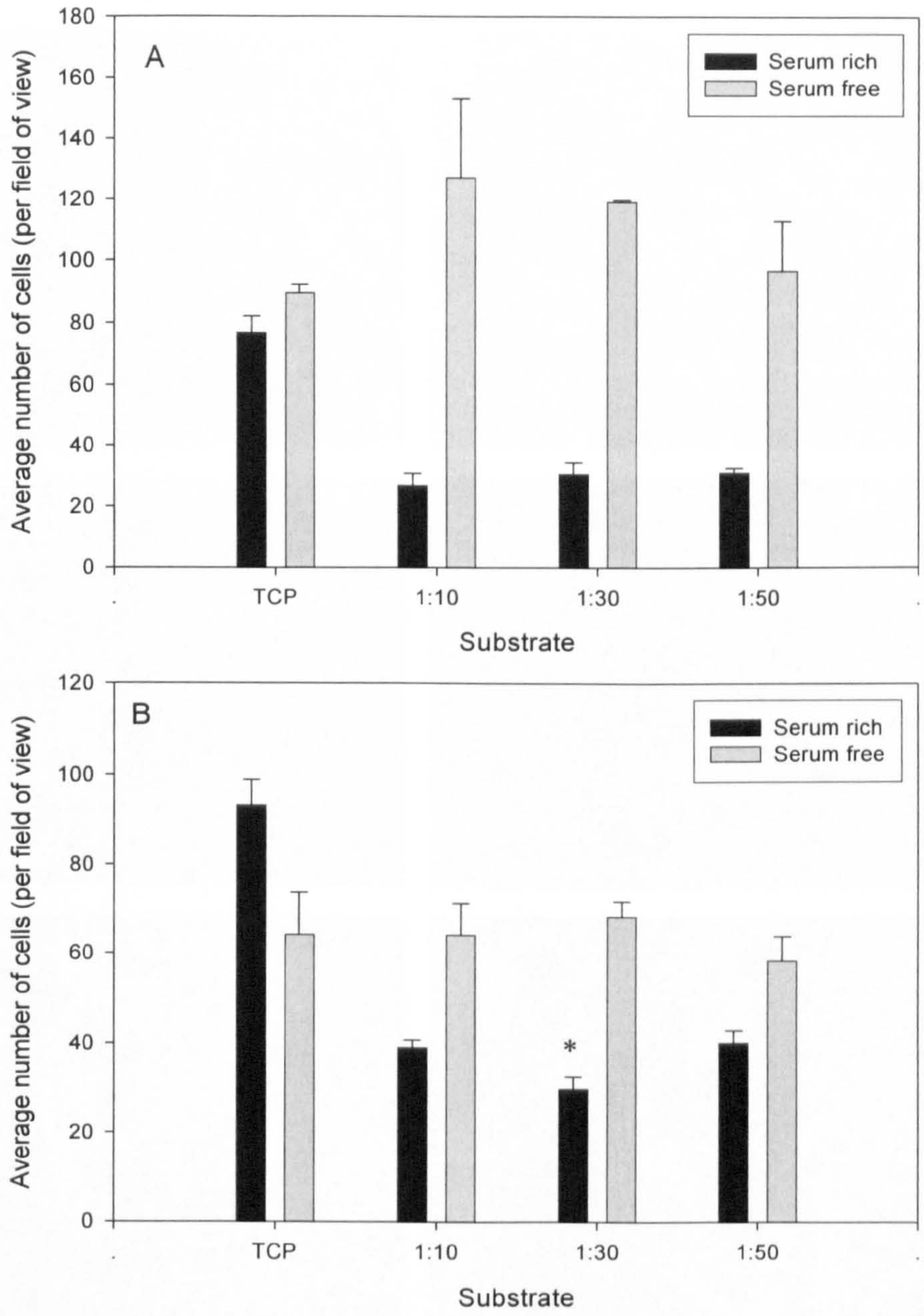


Figure 4-2: Quantification of MSC attachment to uncoated PDMS and TCP after 3 (A) and 24 (B) hours. 2 x 10^5 MSC were seeded onto the PDMS substrates with different crosslinker to base ratios (1:10, 1:30 and 1:50) and a TCP control (2-cm^2 wells) and cultured in either serum rich or serum free growth medium at 37 °C. After 3 and 24 hours the substrates were washed, cells fixed and stained with Dapi before subsequent counting. Numbers are expressed as cells counted per field of view, with 5 fields of view per well and 3 wells counted for each substrate type. * denotes significant difference from corresponding samples.

4.3.3.2 MSC morphology on uncoated PDMS

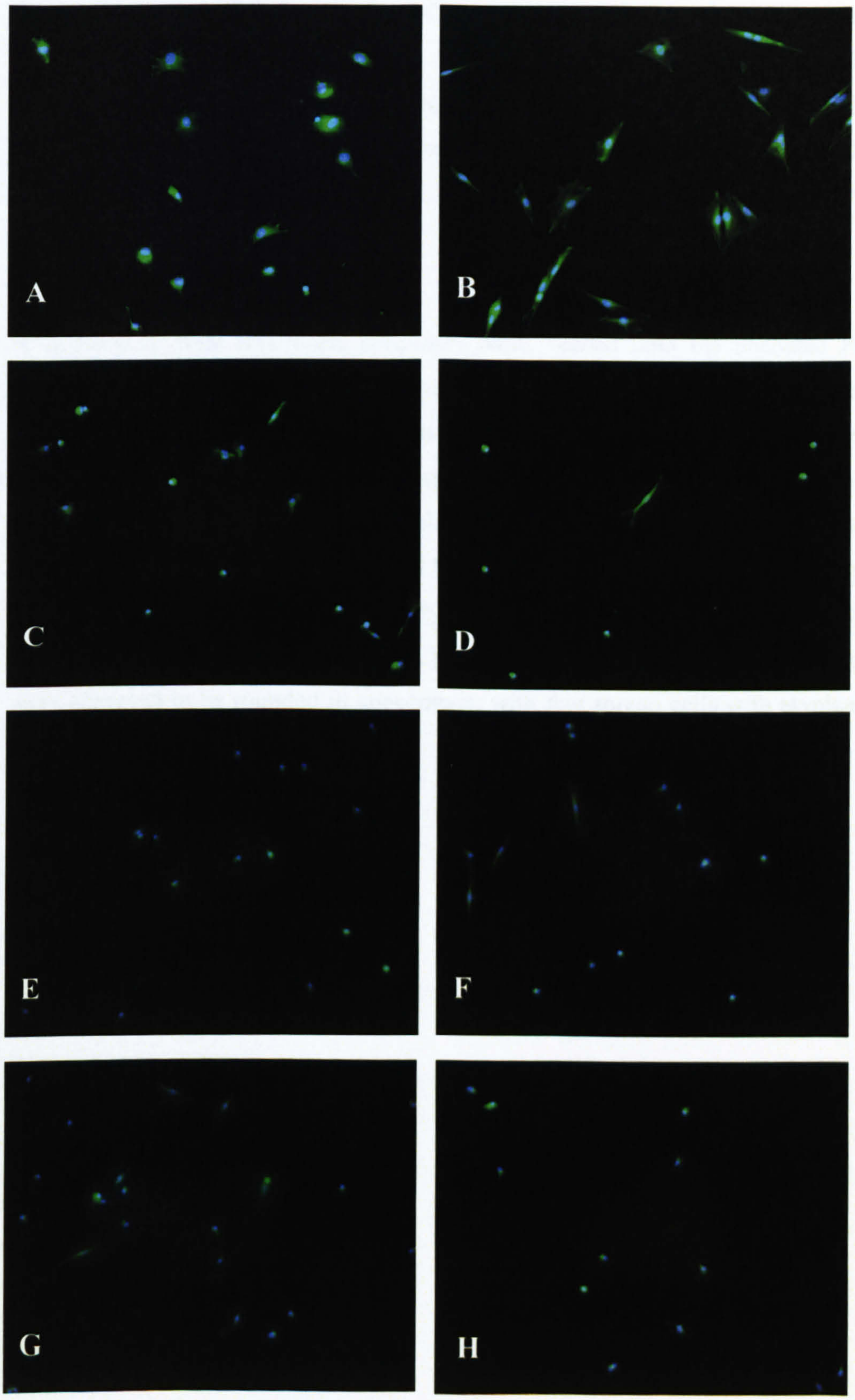


Figure 4-3: Morphology of MSC after 3 and 24 hours culture on uncoated PDMS substrates and a TCP control. 1 x 10^4 cells were cultured on the different substrates in serum rich medium. At 3 and 24 hours cells were fixed and stained with phalloidin (green) and dapi (blue) to show the actin cytoskeleton and nucleus respectively. Images represent the whole cell population for; TCP control 3 hours (A) and 24 hours (B), PDMS 1:10- 3 hours (C) and 24 hours (D), PDMS 1:30- 3 hours (E) and 24 hours (F), PDMS 1:50- 3 hours (G) and 24 hours (H) (Mag x20).

In addition to poor adherence, secondary MSC seeded onto the uncoated PDMS were observed to have an uncharacteristic morphology to that normally observed for MSC in culture on TCP. After 24 hours, the cells appeared rounded in appearance (**Figure 4-3-D,** F, H) compared to the fibroblastic phenotype usually seen **(Figure 4-3-B)**. If the cells were cultured further, by day three abnormal aggregates are seen to form **(Figure 4-4-B)**. This atypical morphology was also observed for primary cells. Under standard culture conditions by day 10 a confluent monolayer is typically seen on TCP with the cells displaying a fibroblastic morphology **(Figure 4-4-C)**. However, cells cultured on the uncoated PDMS were observed to be rounded in appearance, with few spread cells with atypical aggregate formation occurring **(Figure 4-4-D)**.

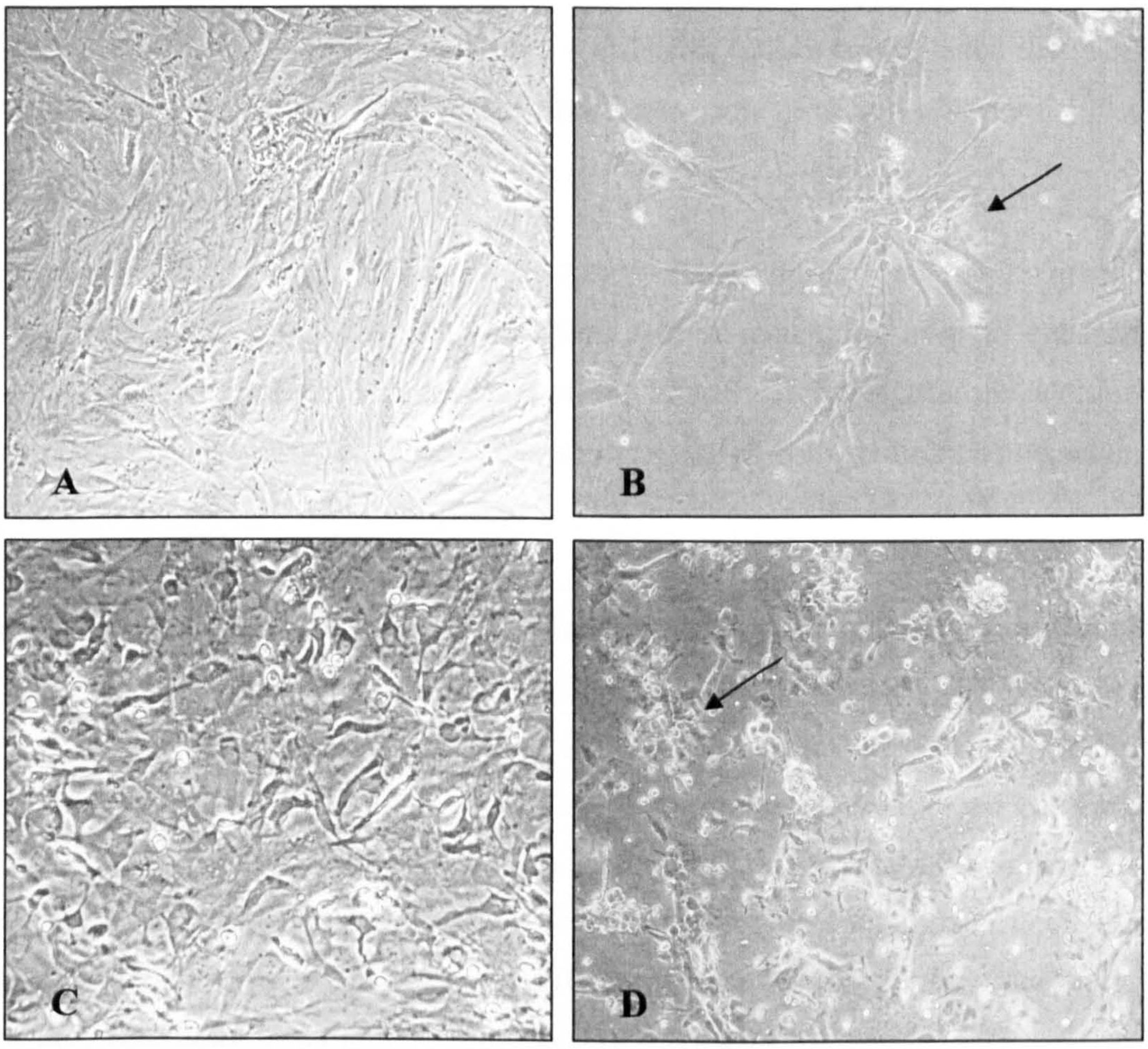


Figure 4-4: Phase contrast light microscope images of MSC cultured on uncoated PDMS (1:30) and TCP. (A) and (B) represent passaged MSC cultured on TCP and uncoated PDMS (1:30) for 10 days whilst (C) and (D) shown the morphology of primary cells on TCP and uncoated PDMS (1:30) after 3 days. The formation of aggregates, atypical of an *in vitro* MSC monolayer culture on TCP, was observed when the cells were cultured on uncoated PDMS substrates. The PDMS images represent all of the uncoated crosslinker: base PDMS substratum (Mag x10).

4.3.3.3 Surface coating

After the comparison was made between the attachment and growth on uncoated PDMS compared to the standard TCP and the observation that the morphology of the cells was atypical of that usually seen in culture on the standard TCP, it was decided that a surface coating was needed to enhance protein adsorption and subsequent cell adhesion.

4.3.3.3.1 Fibronectin

Fibronectin, an ECM protein is one of the two primary adhesion proteins involved in *in vitro* cell attachment in serum rich conditions and is commonly used to enhance cell attachment. MSC attachment was assessed on the PDMS substrates after the addition of a pre-adsorbed fibronectin layer. Attachment was assessed in both serum rich and serum free medium at both 3 and 24-hour time-points.

In serum rich medium, the number of attached MSC was significantly lower on the fibronectin coated PDMS substrates compared to the fibronectin coated TCP control. At the 3-hour time point there were on average 71 % fewer cells on the coated PDMS compared to the coated TCP. By the 24-hour time point slightly more cells had attached but there was still on average 66 % fewer cells on the fibronectin coated PDMS compared to the fibronectin coated TCP. No significant difference was seen on the number of cell attached to the fibronectin coated PDMS except significantly more cells had attached to the 1:10 at the 24-hour time point in serum free conditions (**Figure 4-5**).

In serum free medium the initial attachment of MSC at 3-hours was significantly higher when compared to the serum rich medium and although there was no significant difference seen on the TCP approximately 4 fold more cells had attached under serum free conditions to the PDMS substrates. The difference in attachment between the coated PDMS and TCP was also diminished with an average of 125 ± 7 cells per sample. Although the initial attachment under serum free conditions on the PDMS was high the number of attached cells at the 24-hour time point was significantly less. On average half the number of cells were seen on the different PDMS substrates by the 24-hour time point (**Figure 4-5**).

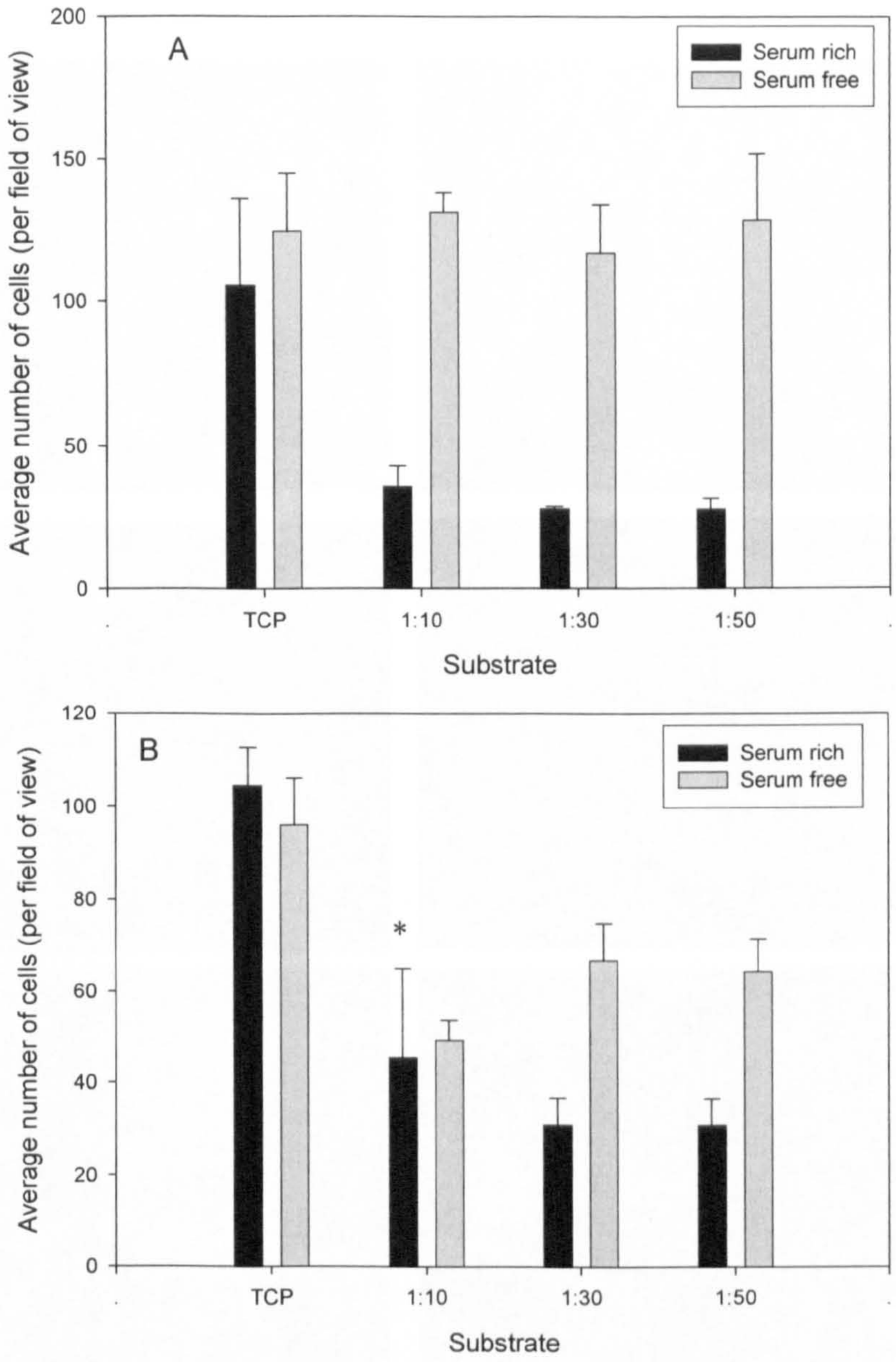


Figure 4-5: Quantification of MSC attachment to fibronectin coated PDMS and a fibronectin coated TCP control after 3 (A) and 24 (B) hours. 20 x 10^4 MSC were seeded onto the different PDMS substrates with different crosslinker to base ratios (1:10, 1:30, 1:50) and a TCP control and cultured in either serum rich or serum free growth medium at 37 °C. After 3 and 24 hours the substrates were washed, cells fixed and stained with Dapi before subsequent counting. Numbers are expressed as cells counted per field of view, with 5 fields of view per well and 3 wells counted for each substrate type. * denotes significant difference from corresponding samples.

4.3.3.4 MSC morphology on fibronectin coated PDMS

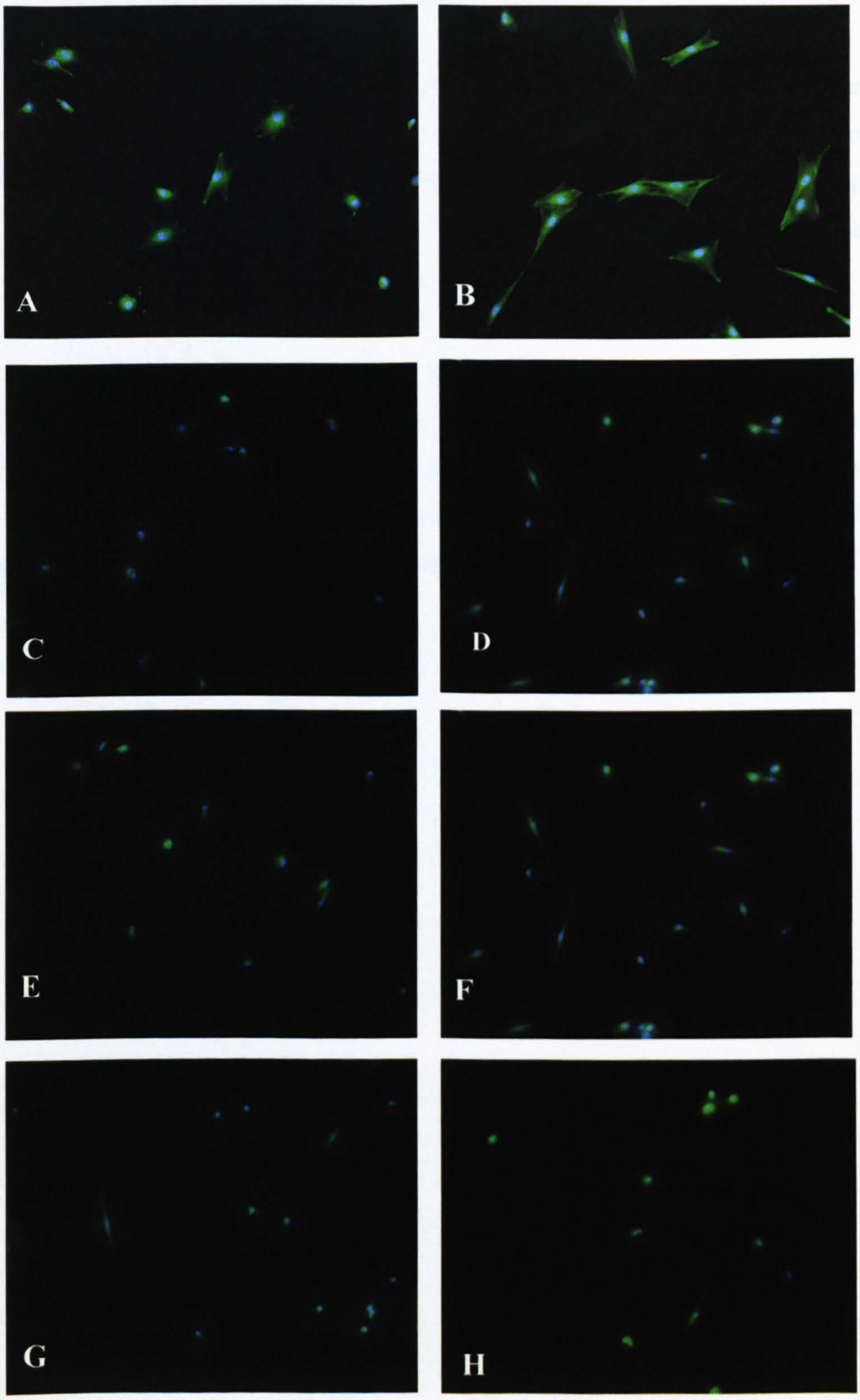


Figure 4-6: Morphology of MSC after 3 and 24 hours culture on fibronectin coated PDMS substrates and a fibronectin coated TCP control. 1 x 10^4 cells were cultured on the diferent subastrates in serum rich medium. At 3 and 24 hours cells were fixed and stained with phalloidin (green) and dapi (blue) to show the actin cytoskeleton and nucleus respectively. Images represent the whole cell population for; fibronectin coated TCP control 3 hours (A) and 24 hours (B), fibronectin coated PDMS 1:10- 3 hours (C) and 24 hours (D), fibronectin coated PDMS 1:30- 3 hours (E) and 24 hours (F), fibronectin coated PDMS 1:50- 3 hours (G) and 24 hours (H). (Mag x20).

Comparable to the images of secondary MSC seeded onto the uncoated PDMS (**Figure 4-4**), MSC seeded onto the fibronectin coated PDMS were observed to have an uncharacteristic morphology to that normally observed for MSC in culture. After 24 hours, the cells appeared rounded in appearance (**Figure 4-6-D, F, H**) compared to the fibroblastic phenotype usually seen on fibronectin coated TCP (**Figure 4-6-B**).

4.3.3.5 Plasma polymerisation coating of the PDMS substrates

Due to the ineffectiveness of the addition of a protein layer in improving cellular attachment and spreading on the PDMS substrates, it was decided that an artificial chemical layer of acrylic acid would be added by employing the technique plasma polymerisation. Before the coating was applied to the PDMS substrates a comparison was made of MSC attachment and growth on TCP with and without an acrylic acid coating to evaluate the suitability of the coating. As shown in **Figure 4.7** there was no significant difference seen between the coated and uncoated TCP in both serum free and serum rich conditions. Furthermore, there was no statistically significant difference in growth over a 7-day period on the acrylic acid coated TCP compared to the standard TCP control (**Figure 4-8**).

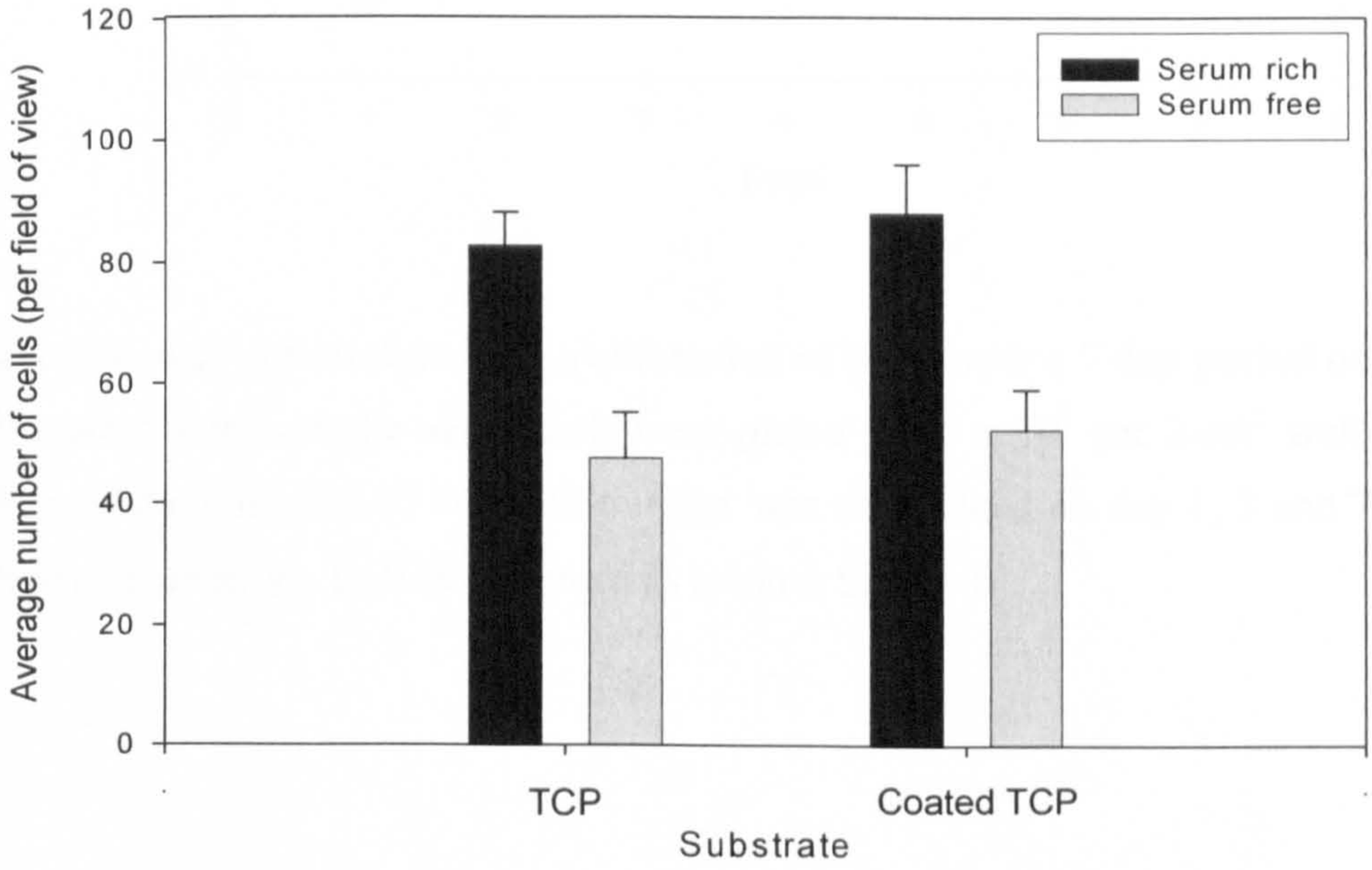


Figure 4-7: Quantification of MSC attachment to TCP and acrylic acid coated TCP after 24 hours. 20 x 10^4 MSC were seeded onto the substrates and cultured in both serum rich and serum free growth medium at 37 °C. After 24 hours the substrates were washed, cells fixed and stained with Dapi before subsequent counting. Numbers are expressed as cells counted per field of view, with 5 fields of view per well and 3 wells counted for each substrate type.

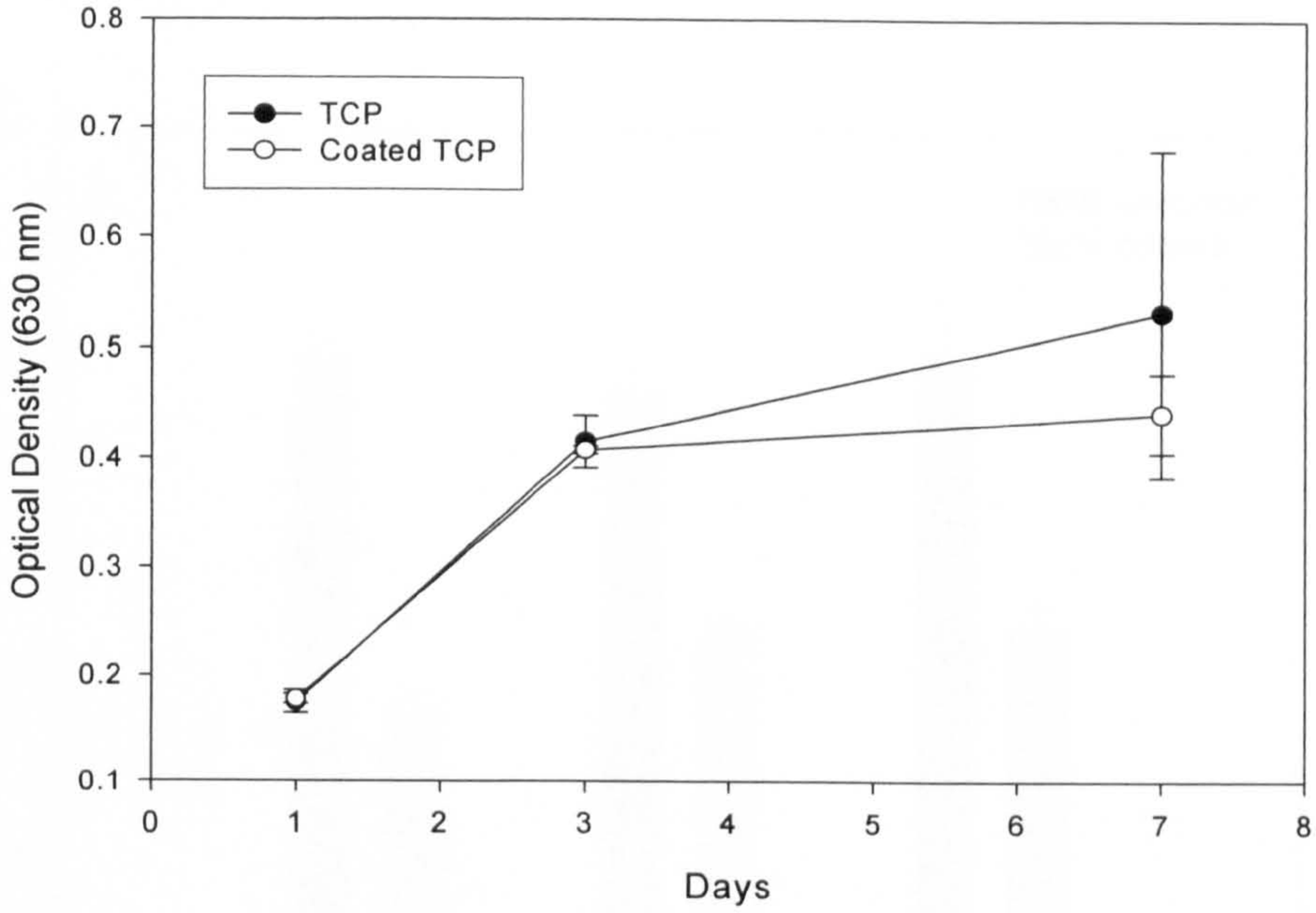


Figure 4-8: A graph to show the proliferation of MSC over a 7 day period on TCP and TCP coated with acrylic acid. Cells were plated out 2 x 10^4 per 2-cm^2 well in growth medium and cultured at 37 °C. Cell number was determined on day 1, 3 and 7 using the methylene blue assay. Data is presented as mean ± SD (N=6).

4.3.4 Surface Characterisation

4.3.4.1 Contact angle

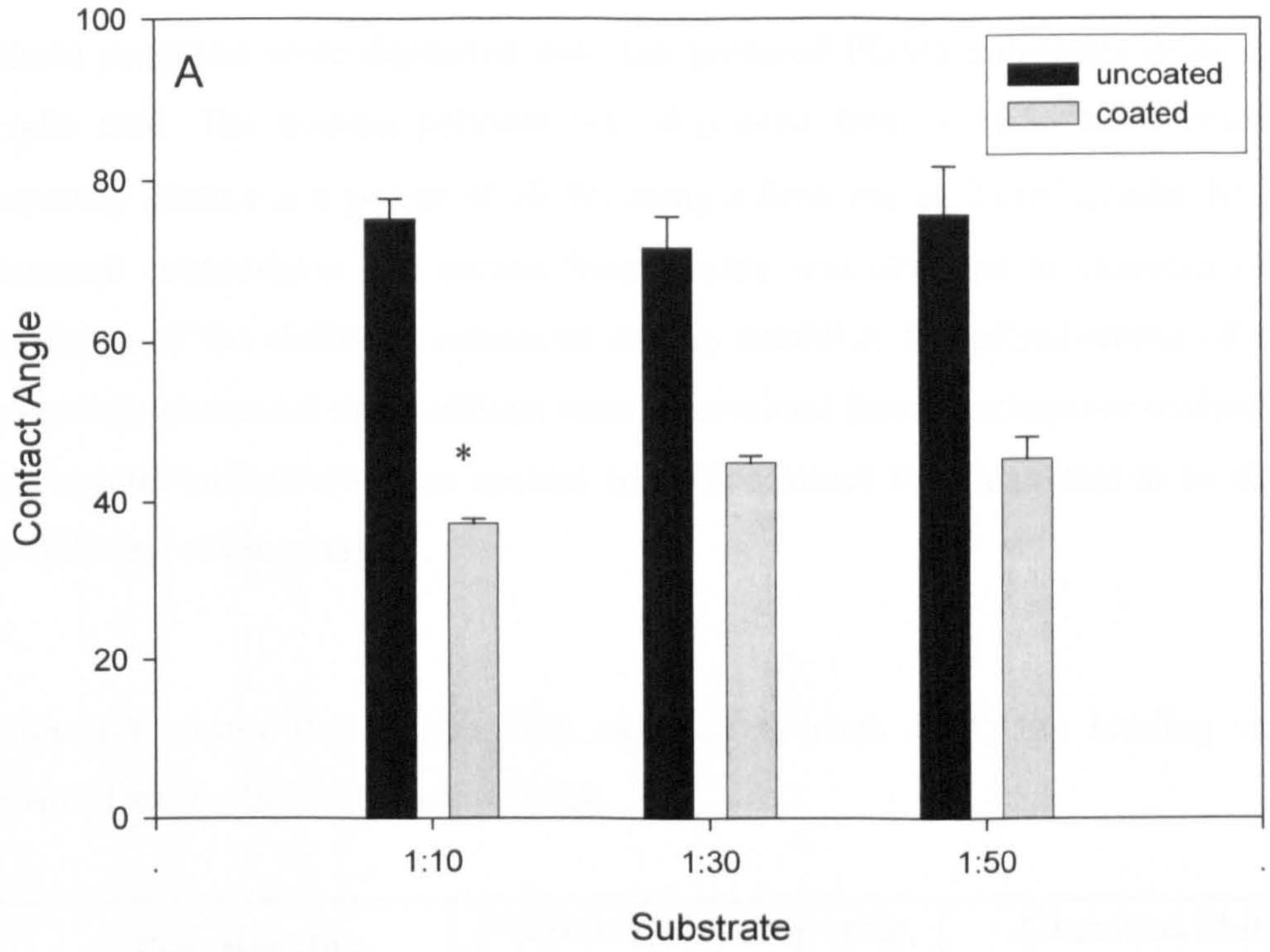


Figure 4-9: Static contact angles for PDMS substrata before and after plasma polymerisation coating with acrylic acid. Data are shown for three oligomer to crosslinker ratios (1:10, 1:30 and 1:50) (n=5). * denotes significant difference from corresponding samples with in the same group.

To determine the hydrophobicity of the different PDMS substrates contact angle measurements were performed. The data confirms that untreated PDMS is hydrophobic and therefore will resist protein adsorption from solution. The addition of a layer of acrylic acid creates a hydrophilic layer, enables protein adsorption and therefore cell adhesion. The addition of the acrylic acid reduces the contact angle from an average of 75° $\pm$ 5° to 43° $\pm$ 3° (**Figure 4-9**). No significant difference in contact angle was seen on the uncoated PDMS, regardless of cross-linker concentrations. However, after the PDMS had been coated with acrylic acid, a slight increase in the contact angle measurement was observed,

as the substrate got softer with the 1:10 PDMS being 9° and 8° lower than the 1:30 and 1:50 respectively.

4.3.4.2 XPS

Plasma polymers were deposited onto the prepared PDMS substrates using the monomer acrylic acid. The plasma polymer was deposited from a 13.56 MHz continuous wave frequency plasma at a power of 10 W, using a flow rate of 2 cm^3 $_{stp}$/min. XPS analysis of elemental composition and carbon functionality was obtained to characterise the surface chemistry of the different substrates and to establish the effectiveness of the coatings. Percentage elemental compositions were determined through computer analysis of the wide scan spectra and narrow scan spectra analysis allowed functionalities to be determined by curve fitting of the peaks.

Table 4-1 shows the functionality assigned to each peak, the binding scale and the chemical shift relative to the C-C peak.

Functionality	Position on binding scale, (eV)	Chemical Shift (relative to C-C, (eV)
C-Si	284.6	- 0.4
CHx	285	0
C-O	286.5	+ 1.5
O-C=O	289	+ 4

Table 4-1: A table to show the position on binding scale, chemical shift relative to C-C and the functionality assigned to XPS narrow scan spectra determined by curve fitting the peaks.

Figure 4-12 shows representative wide scan spectra for each of the uncoated substrates (1:10, 1:30 and 1:50). The spectra contain peaks arising from carbon, oxygen and silicon. Calculation of the % elemental composition for each of the spectra shows that there is no difference between the uncoated substrates and that changing the degree of crosslinking does not affect the surface elemental composition of the different samples. The surface

chemistry for each sample is typically 44 % carbon, 27 % oxygen and 29 % silicon **Figure 4-10**. Theoretical values for PDMS are 50 % carbon, 25 % silicon and 25 % oxygen. (Briggs and Seah., 1990). A representative narrow scan spectrum is shown in **Figure 4-11** and demonstrates the presence of a carbon peak with no acid shoulder.

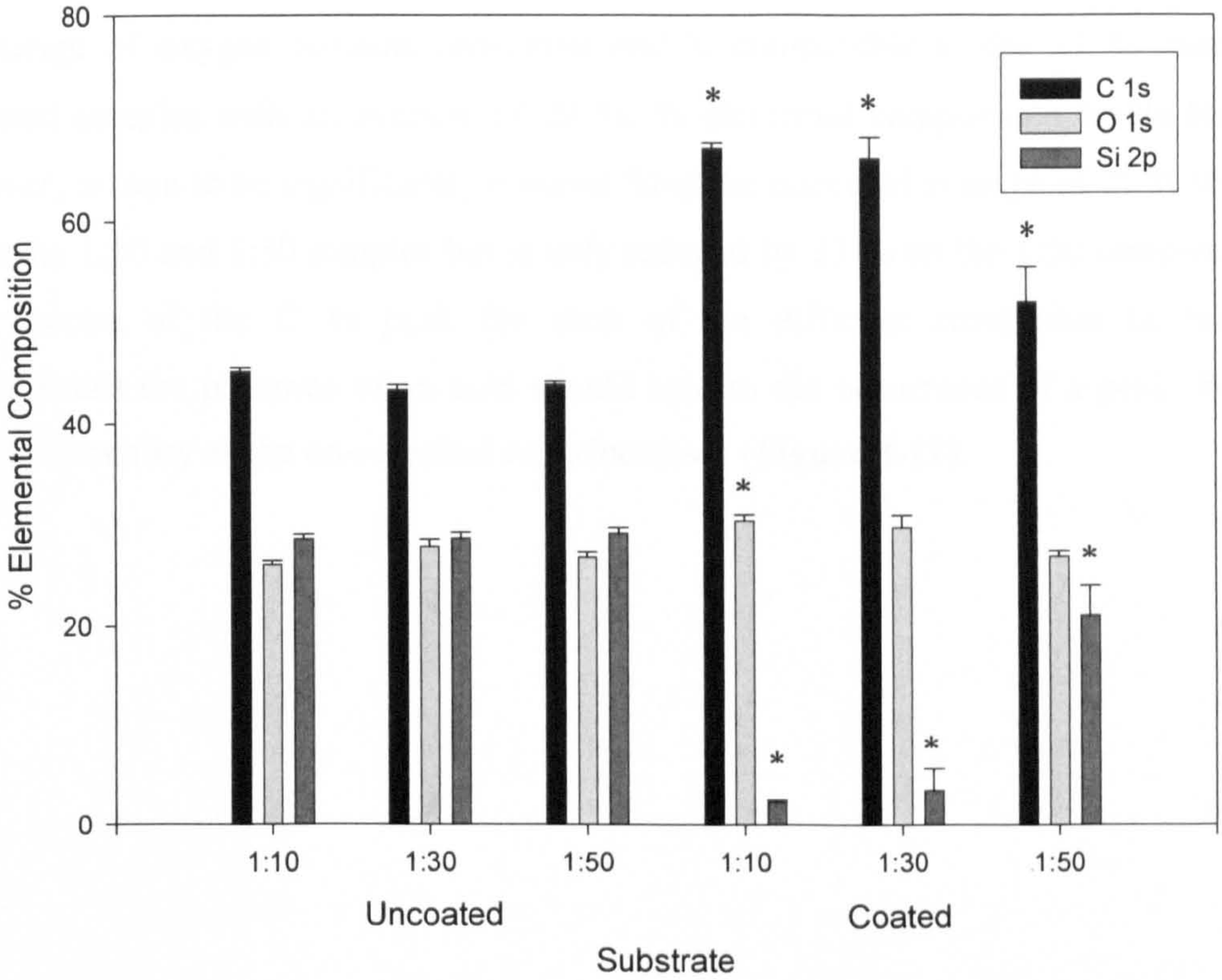


Figure 4-10: % Atomic elemental Composition for PDMS substrates with different crosslinking densities before and after coating with plasma polymerised acrylic acid (n=3). * denotes significant difference from uncoated control.

Figure 4-13 shows representative wide scan spectra for each of the coated substrates (1:10, 1:30 and 1:50). The spectra contain peaks arising from carbon, oxygen and also from silicon. The silicon peaks are seen to be very small in the 1:10 and 1:30 but is considerably larger in the 1:50. Percentage elemental composition data in **Figure 4-10** shows that coating the PDMS substrates with acrylic acid raises the percentage of carbon by an average 51 % for the 1:10 and 1:30 but only by an average 18 % for the 1:50 substrate. The percentage of oxygen remains consistent and is comparable to the 27 % seen for the uncoated samples with an average of 29 %. % elemental composition levels for silicon, however, is seen to be significantly reduced from the uncoated average of 29 % to below 4 % for the 1:10 and 1:30 samples but is only reduced by 21 % on the 1:50 samples. Narrow scan spectra of the C 1s peak for each of the different crosslinker to base ratios demonstrates the presence of an acid should lead to the occurrence of a peak shift in the acid functionality as the cross-linked ratio increases **(Figure 4-11)**.

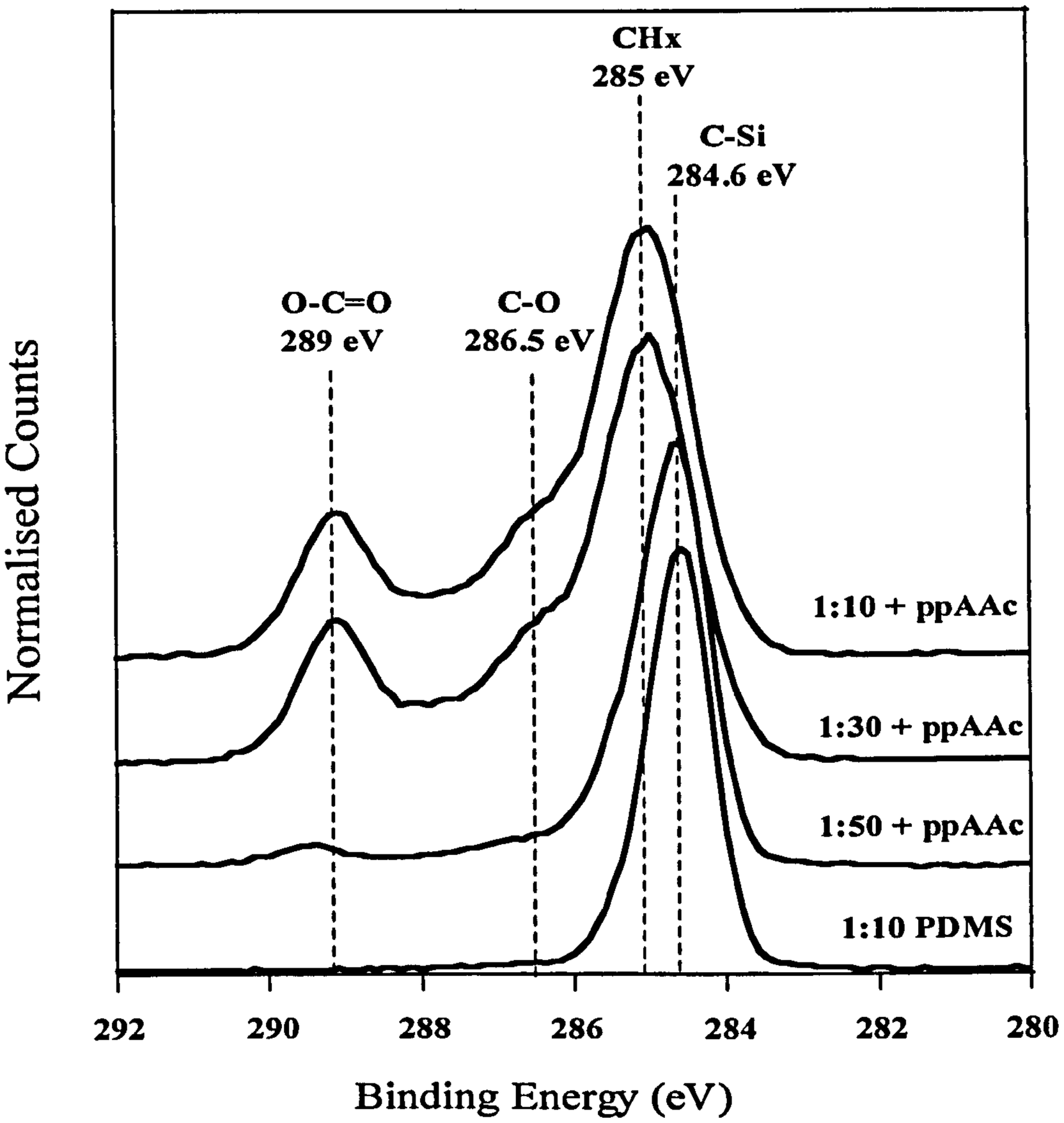


Figure 4-11: Typical XPS narrow scan spectra from plasma polymerised acrylic acid deposited on to PDMS substrates.

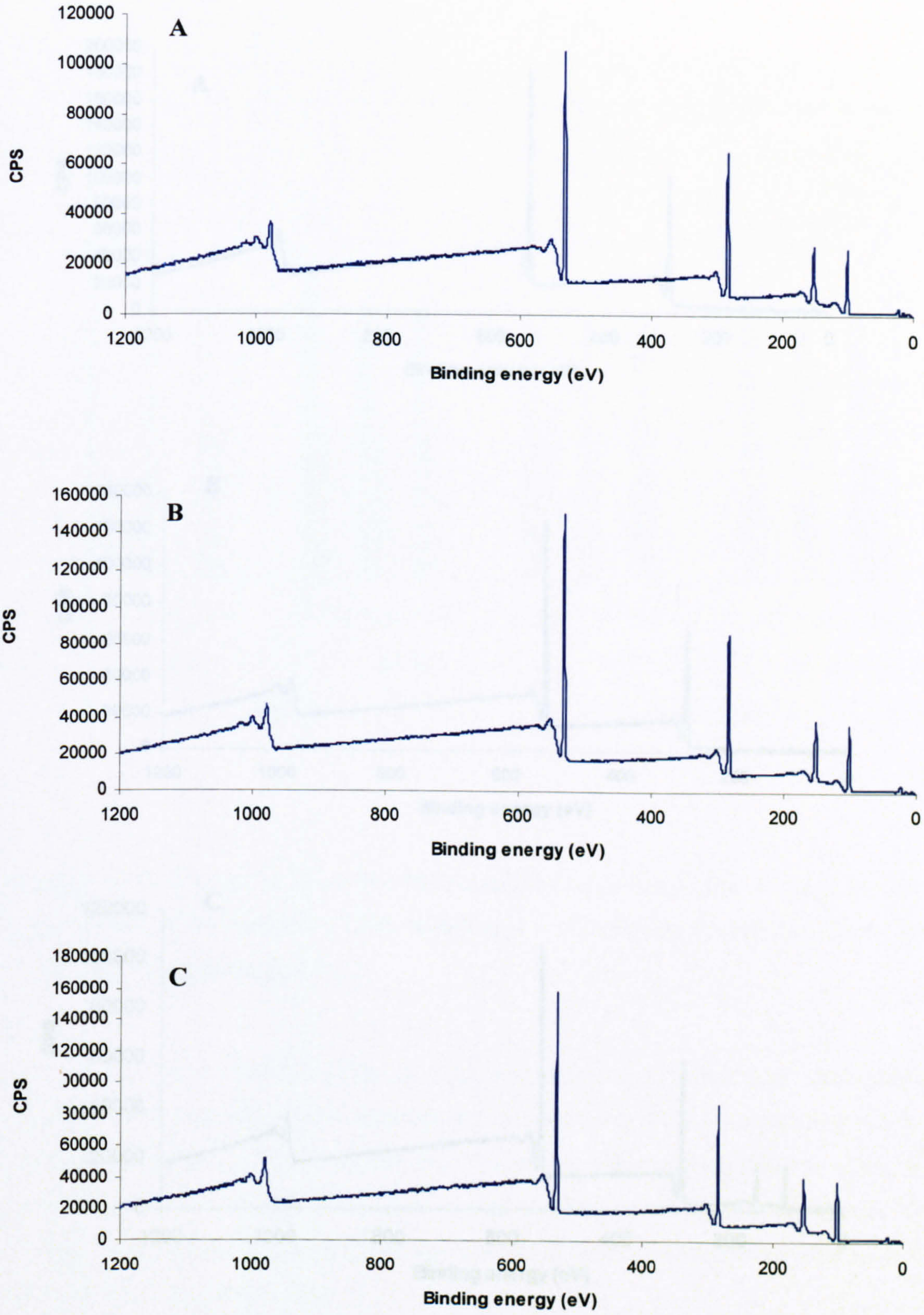


Figure 4-12: Typical XPS wide scan spectra for Uncoated PDMS samples with varying crosslinker to base ratios 1:10 (A), 1:30 (B) and 1:50 (C).

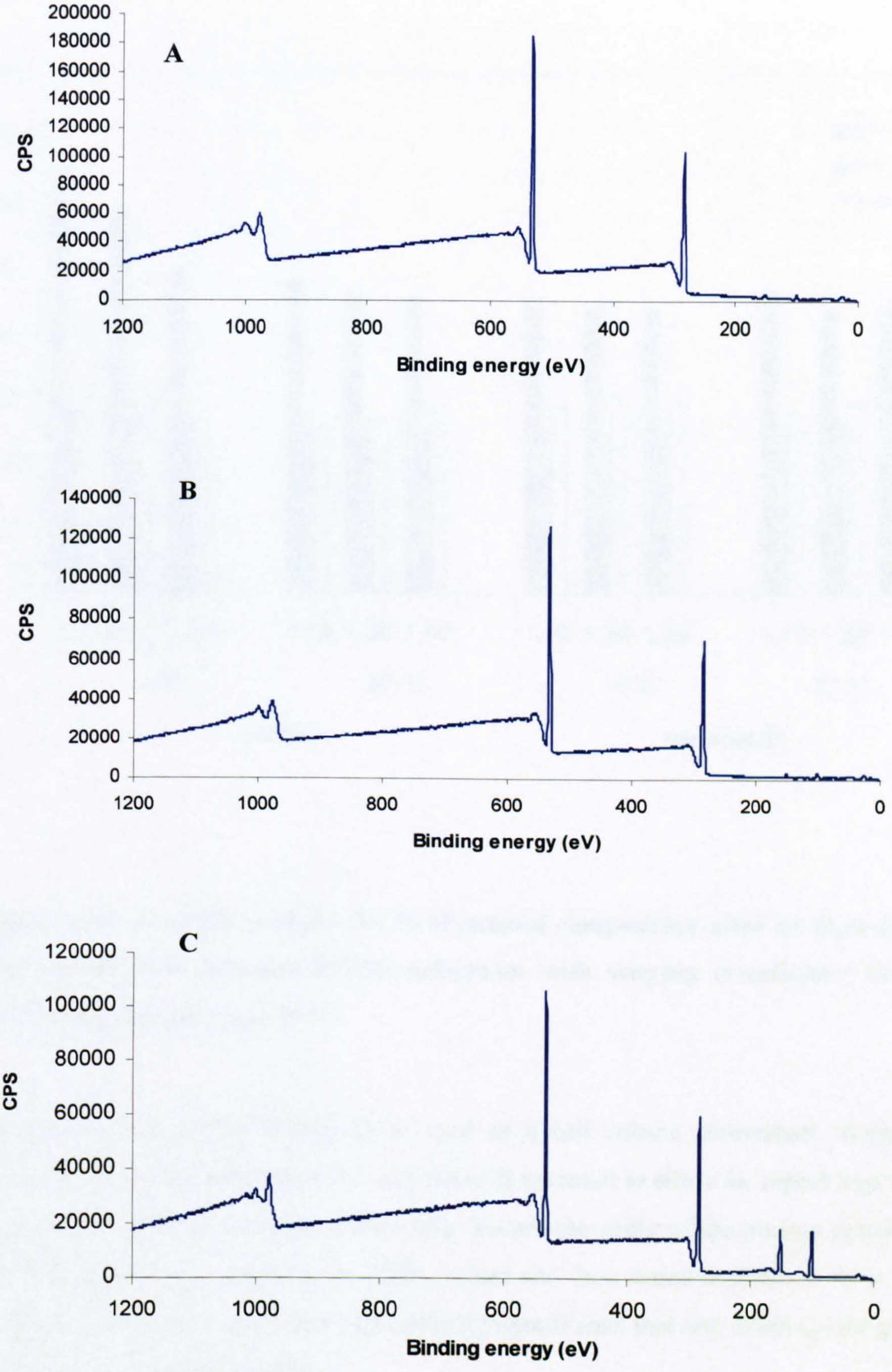


Figure 4-13: Typical XPS wide scan spectra for acrylic acid coated PDMS samples with varying crosslinker to base ratios 1:10 (A), 1:30 (B) and 1:50 (C).

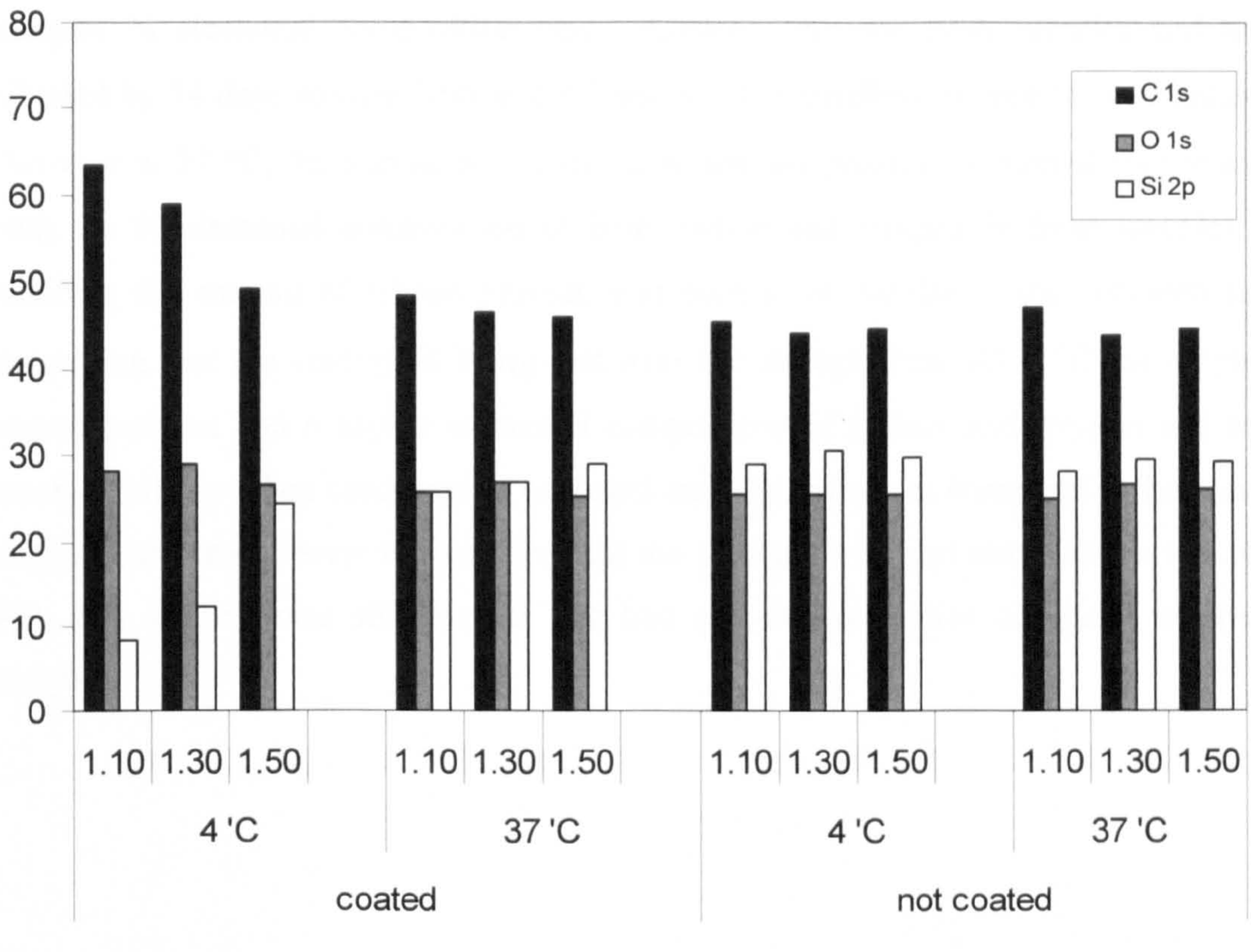


Figure 4-14: A graph to show the % elemental composition after 14 days for acrylic acid coated and uncoated PDMS substrates with varying crosslinker: base ratios (1:10-1:50) at both 4 and 37°C.

For acrylic acid coated PDMS to be used as a cell culture substratum, storage of the polymers once deposition has been completed is essential to allow for repeat experiments to be made using the same batch of substrate. To test the aging of the plasma polymer on the different substrates, samples were made, coated and then stored at either 4 °C or 37 °C for 14 days. Uncoated samples were also stored to see if time had any effect on the polymer at the different crosslinker ratios.

Storage of the acrylic acid coated PDMS polymers for 14 days affected the % elemental composition on the different substrates at both temperatures investigated. The uncoated samples % elemental composition was consistent with the fresh samples and was not affected by 14 days storage both at 4 °C and 37 °C regardless of base to crosslinker ratio. However at 37 °C, the acrylic acid coating, which has previously been shown to increase both the % elemental composition of both carbon and oxygen in fresh samples whilst reducing the amount of silicon present, was seen to be similar to the uncoated samples suggesting that the coating is being lost over the storage time. At 4 °C the acrylic acid coated samples had a higher elemental composition of carbon and oxygen and reduced levels of silicon when compared to uncoated samples. However compared to fresh samples oxygen and carbon levels were reduced for the 1:30 and 1:50 and more silicon was present. The 1:10 coating was still present and had not changed when compared to the fresh samples.

4.3.4.3 Mechanical testing

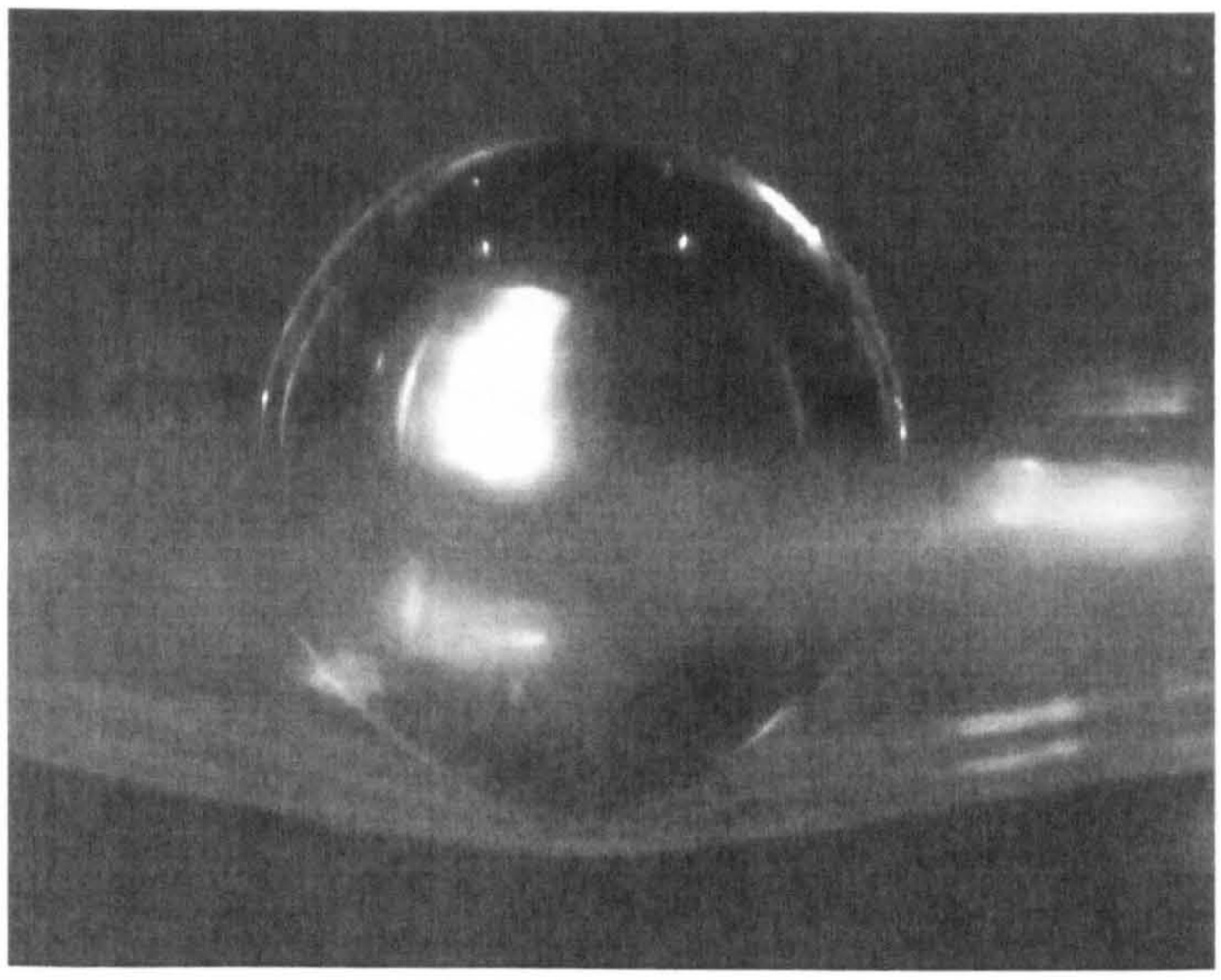


Figure 4-15: An image to show the indentation of the PDMS membranes by a smooth sphere of known mass.

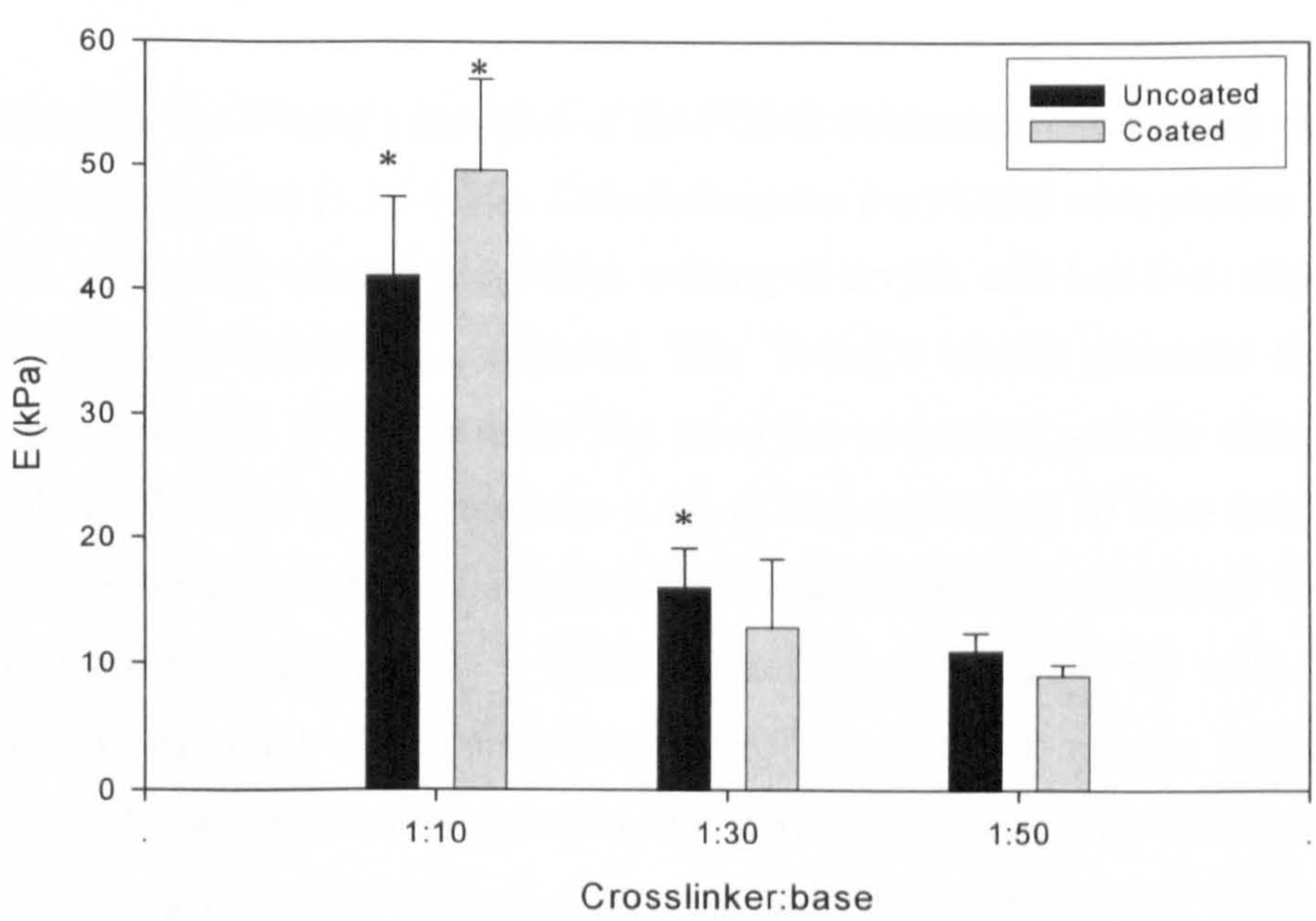


Figure 4-16: Elastic moduli determined by indentation of the PDMS substrata. The graph shows the Young's modulus (E) for three crosslinker to base ratios (1:10, 1:30 and 1:50). Data are shown as the mean of 5 samples ± SD. * denotes significant difference from next sample in the corresponding group.

In order to determine whether the mechanical properties of the underlying substratum have effects on cellular behaviour it is imperative that an accurate Young's modulus of the substrata is measured. Although the tensile testing measurements enabled bulk Young's modulus data to be calculated **Figure 4-1,** further characterisation using a lower percentage strain was necessary to record the moduli on a localised scale. Working in collaboration with Dr. Isaac Lui, moduli were calculated relevant to the cells local microenvironment and enabled consideration of any heterogeneities in the substrate.

Using a high magnification, long distance (up to 120 times) microscope and associated digital analysis software, clear images of the deformation profile of the stainless steel ball on the PDMS substrates were obtained **(Figure 4-15).** Due to the clarity of these images, depth of indentation for each sample was easily and accurately measured using computer software. This microscopic system was also used to measure the thickness of the PDMS (typical <1 mm) enabling accurate, reproducible measurements. The values obtained allowed calculation of the Young's modulus for each of the samples.

The values for the Young's modulus of the PDMS substrates with varying oligomer to base ratios were calculated (1:10-1:50). Calculations for the PDMS after plasma polymerisation had been performed where a nano-thick coating of acrylic acid had been deposited onto the PDMS substrates were also completed. The Young's moduli obtained for the different substrates are shown in **Figure 4-16.** The trend was consistent with the calculated uncoated PDMS bulk Young's moduli **(Figure 4-1);** as the crosslinker to base ratio increased the Young's modulus decreased. Using this technique the moduli calculated for the uncoated samples were an average of 41 ± 6, 16 ± 3 and 11 ± 1 kPa for the 1:10, 1:30 and 1:50, respectively. Although slight differences were seen between plasma polymerised coated and uncoated samples the difference was not statistically significant with average moduli of 50 ± 1, 13 ± 1 and 9 ± 2 kPa for the 1:10, 1:30 and 1:50 respectively.

4.3.5 Evaluation of acrylic acid coating for cell culture

4.3.5.1 Attachment

To determine whether PDMS cross-linker concentration affects the attachment of MSC after both a 3 and 24-hour time period, cells were seeded at a concentration of 2×10^4 and then the number of attached cells determined by 'Dapi' staining and subsequent automated counting.

At the 3-hour point, in serum rich medium there was no difference in the number of attached cells with an average of 74 ± 4 cells per field of view for both the different PDMS substrates and the acrylic acid coated TCP control. At the 24-hour time point the number of attached cells on the PDMS substrates had increased by 22 % to an average of 90 ± 5 cells per field of view, whereas on the acrylic acid coated TCP there was no increase with the number of attached cells remaining constant at 78. Regardless to the difference in time of attachment, there was no significant difference between the number of cells attached to the PDMS and the acrylic acid coated TCP at either of the time points.

In the serum free medium at the 3-hour time it was observed that an average of 27 % more cells were attached to all of the different substrates when compared to the number of cells attached under serum rich conditions. However, the 24-hour time point saw fewer cells on the substrates suggesting that under serum free conditions the initial attachment seen at 3 hours was unsustainable. As observed in the serum rich conditions, no significant differences were seen between the numbers of cells attached at either of the time point on the different substrates and therefore cross linker concentration and hence the Young's modulus of the underlying does not have an effect on cell attachment.

4.3.5.2 Morphology

Cells cultured on PDMS coated with acrylic acid looked morphologically typical of MSC in culture on TCP on the 1.10 substrate but the cells did not appear to be as fibroblastic in appearance on the 1:30 and 1:50 PDMS substrates **(Figure 4-18)**.

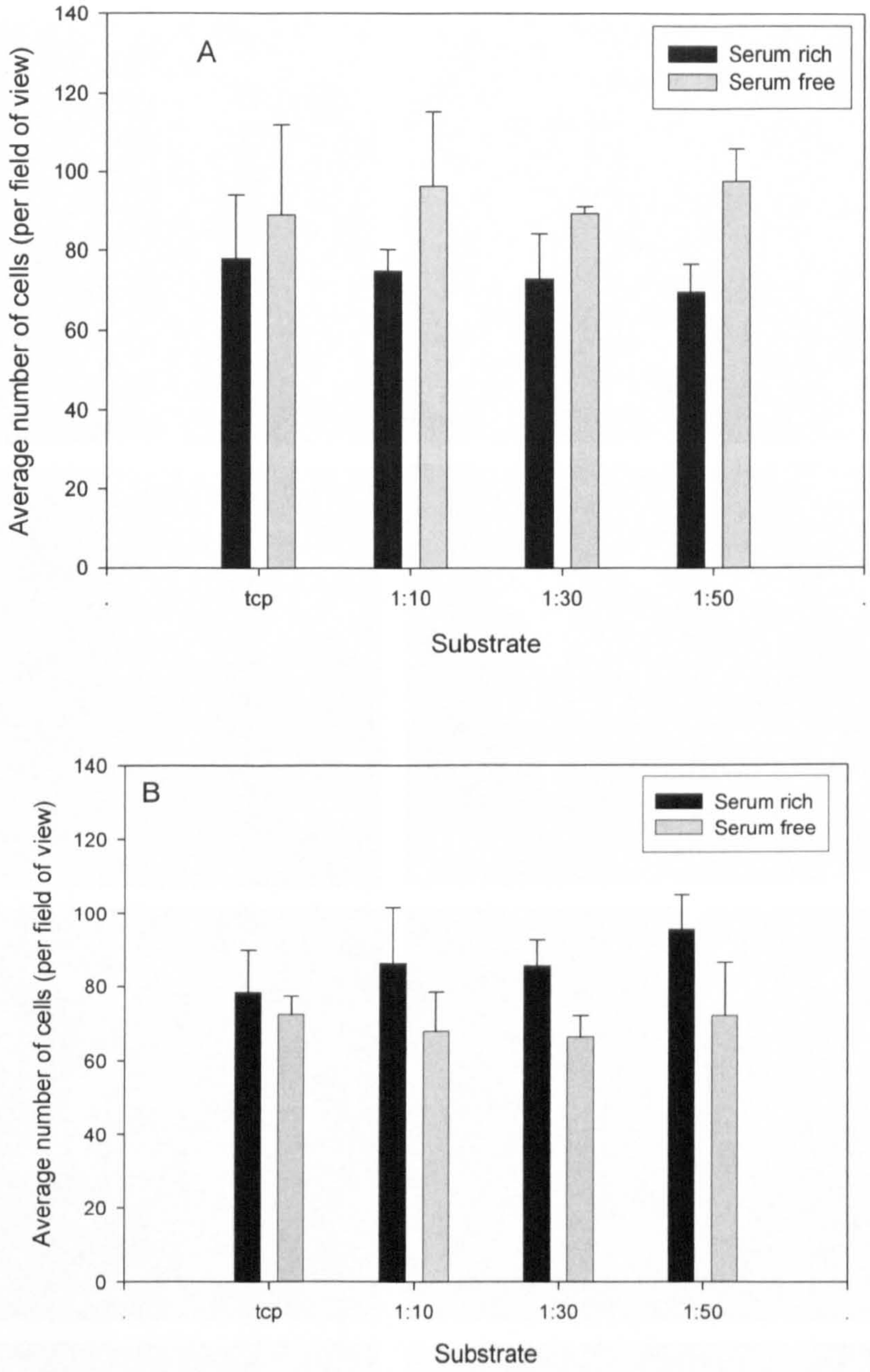


Figure 4-17: Quantification of MSC attachment to acrylic acid coated PDMS and TCP after 3 and 24 hours. 2 x 10^5 MSC were seeded onto coated PDMS substrates with different crosslinker to base ratios (1:10, 1:30, 1:50) and a coated TCP control and cultured in both serum rich and serum free growth medium at 37 °C. After 3 (A) and 24 (B) hours the substrates were washed, cells fixed and stained with Dapi before subsequent counting. Numbers are expressed as cells counted per field of view, with 5 fields of view per well and 3 wells counted for each substrate type.

4.3.5.3 Morphology of MSC cultured on acrylic acid coated substrates

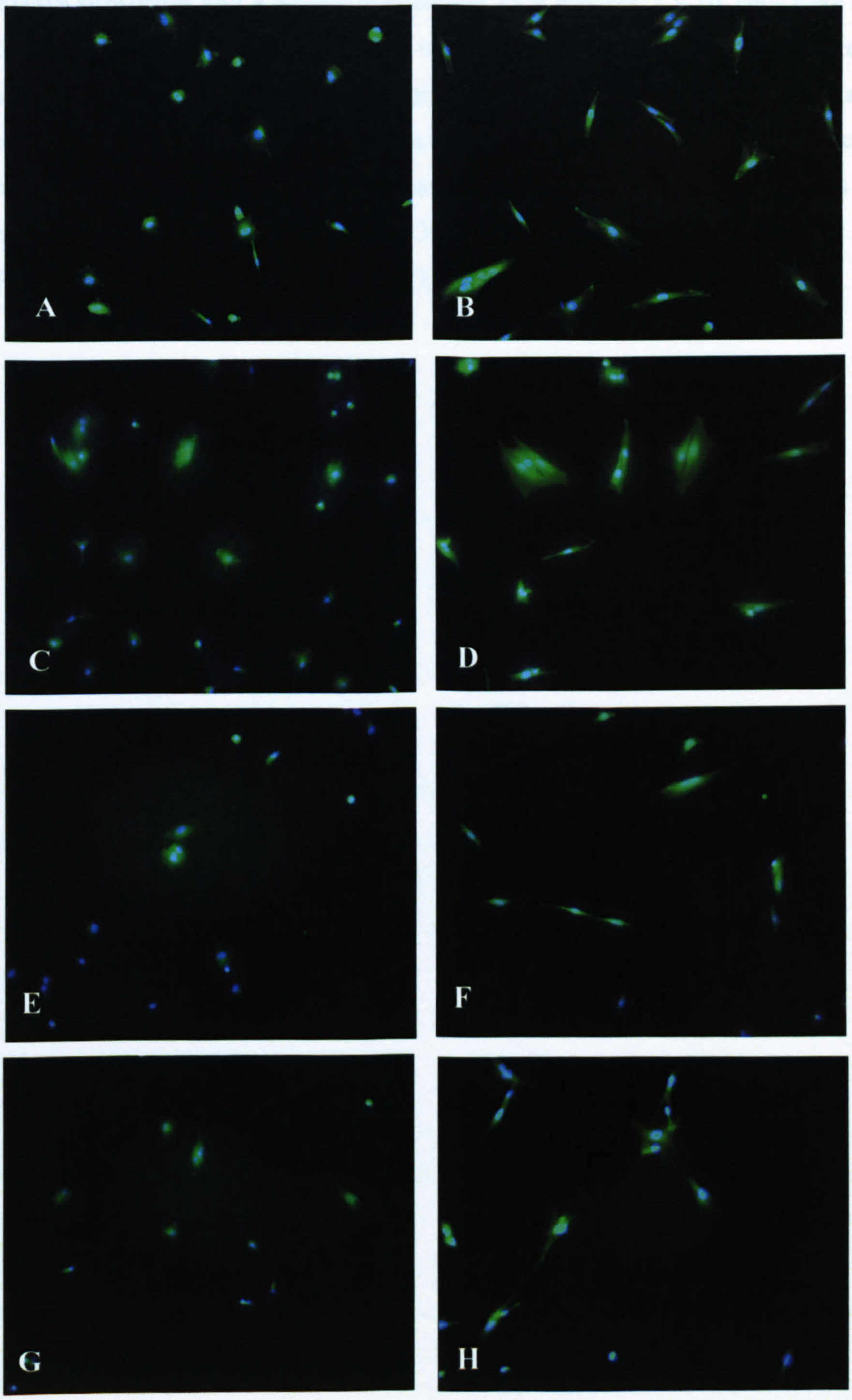


Figure 4-18: Morphology of MSC after 3 and 24 hours culture on acrylic acid coated PDMS substrates and a acyrlic acid coated TCP control. 1 x 10^4 cells were cultured on the diferent subastrates in serum rich medium. At 3 and 24 hours cells were fixed and stained with phalloidin (green) and dapi (blue) to show the actin cytoskeleton and nucleus respectively. Images represent the whole cell population for; acrylic acid coated TCP control 3 hours (A) and 24 hours (B), acrylic acid coated PDMS 1:10- 3 hours (C) and 24 hours (D), acrylic acid coated PDMS 1:30- 3 hours (E) and 24 hours (F), acrylic acid coated PDMS 1:50- 3 hours (G) and 24 hours (H). (Mag x 20).

4.3.6 Evaluation of acrylic acid coated PDMS for the study of substrate stiffness on cell behaviour

4.3.6.1 Effects of substrate stiffness on MSC proliferation

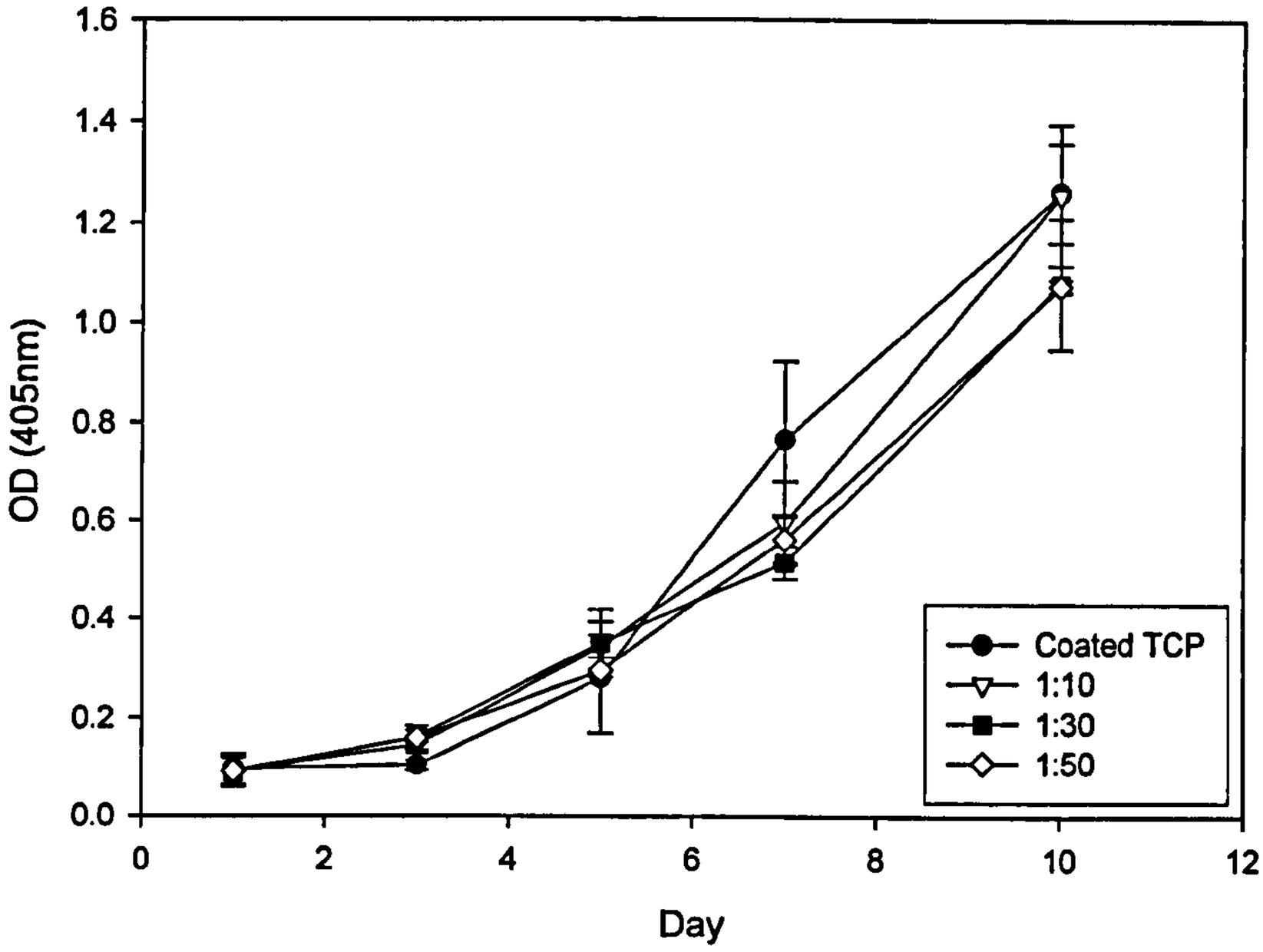


Figure 4-19: A graph to show the proliferation of MSC over a 10 day period on PDMS substrates with a range of Young's moduli ranging from 12 Kpa (softest) to 1 MPa (hardest). Cells were plated out 1 x 10^4 per 2-cm^2 well in growth medium and cultured at 37 °C. Cell number was determined on different days as indicated using the methylene blue assay. Data is presented as mean ± SD (N=5), representative of three individual experiments.

Cell proliferation was assessed by determining the number of cells on the different PDMS substrates (1:10,1:30 and 1:50) and an acrylic acid coated TCP control, over a 10-day period. As shown in **Figure 4-19,** it was seen that cell proliferation was highest on acrylic acid coated TCP and on the PDMS with the highest Young's modulus i.e. the hardest substrate. Growth on the 1.30 and 1:50 was slightly slower although this was not significant. The initial reading taken on day 3 confirms that cell attachment is constant

regardless to the different substrate used and confirms the attachment data seen in **Figure 4-17.**

4.3.6.2 Effects of substrate stiffness on the viability of cultured MSC

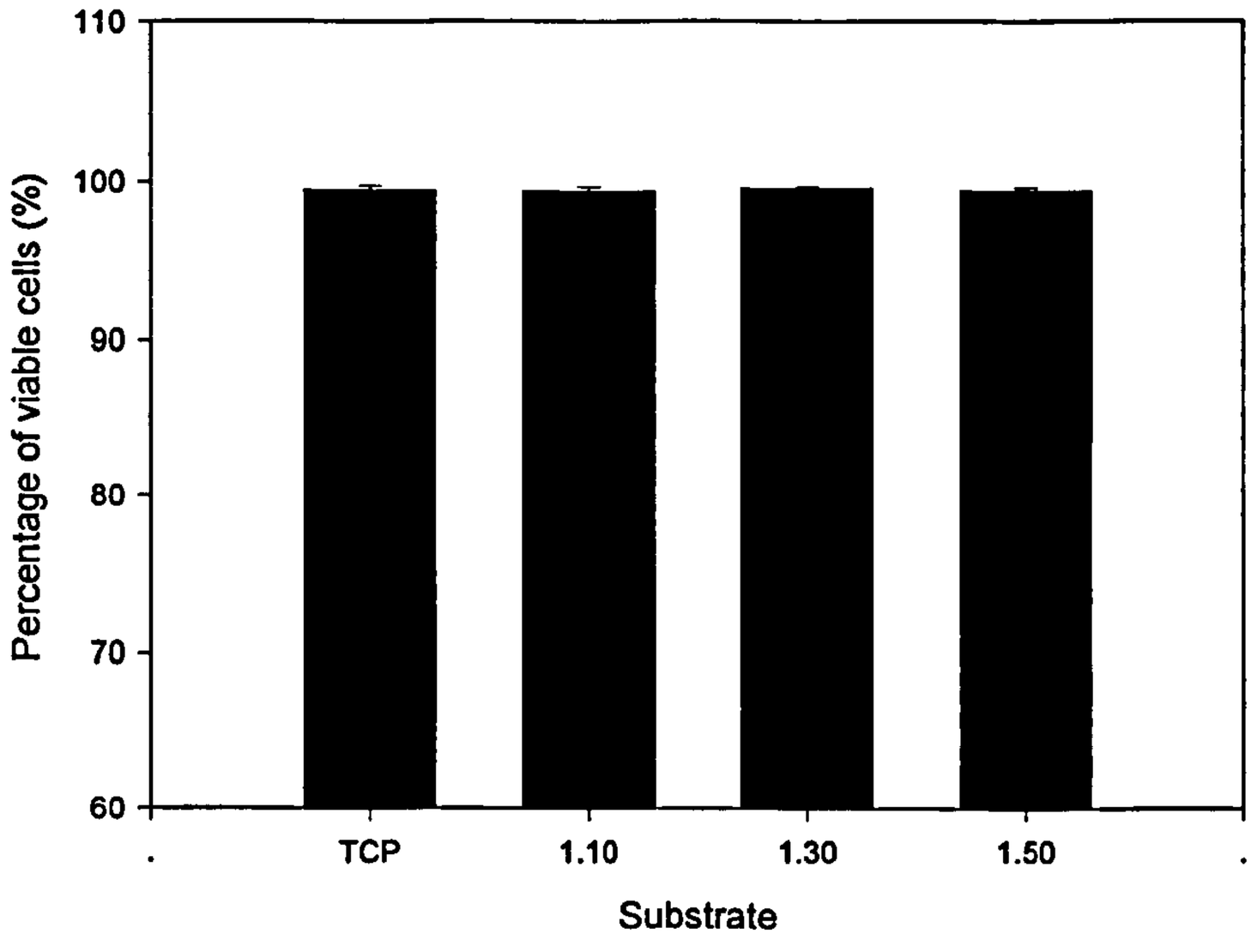


Figure 4-20: Effects of substrate stiffness on viability in cultured MSC. 300 µl of primary MSC were plated out per 2-cm^2 well on PDMS substrates (1:10, 1:30 and 1:50) and coated TCP control in growth medium and cultured at 37 °C for 7 days. Cell viability was determined using the Guava Viacount assay with typically 1000 events read. Data is presented as mean ± SD, representative of three individual experiments (n=3).

The percentage of viable cells was observed to be consistent despite the change in stiffness of the underlying substrate. An average of 99.5 % of the total population were viable regardless of substrate type and crosslinker to base ratio (**Figure 4-20**).

4.3.6.3 Effects of substrate stiffness on apoptosis levels in cultured MSC

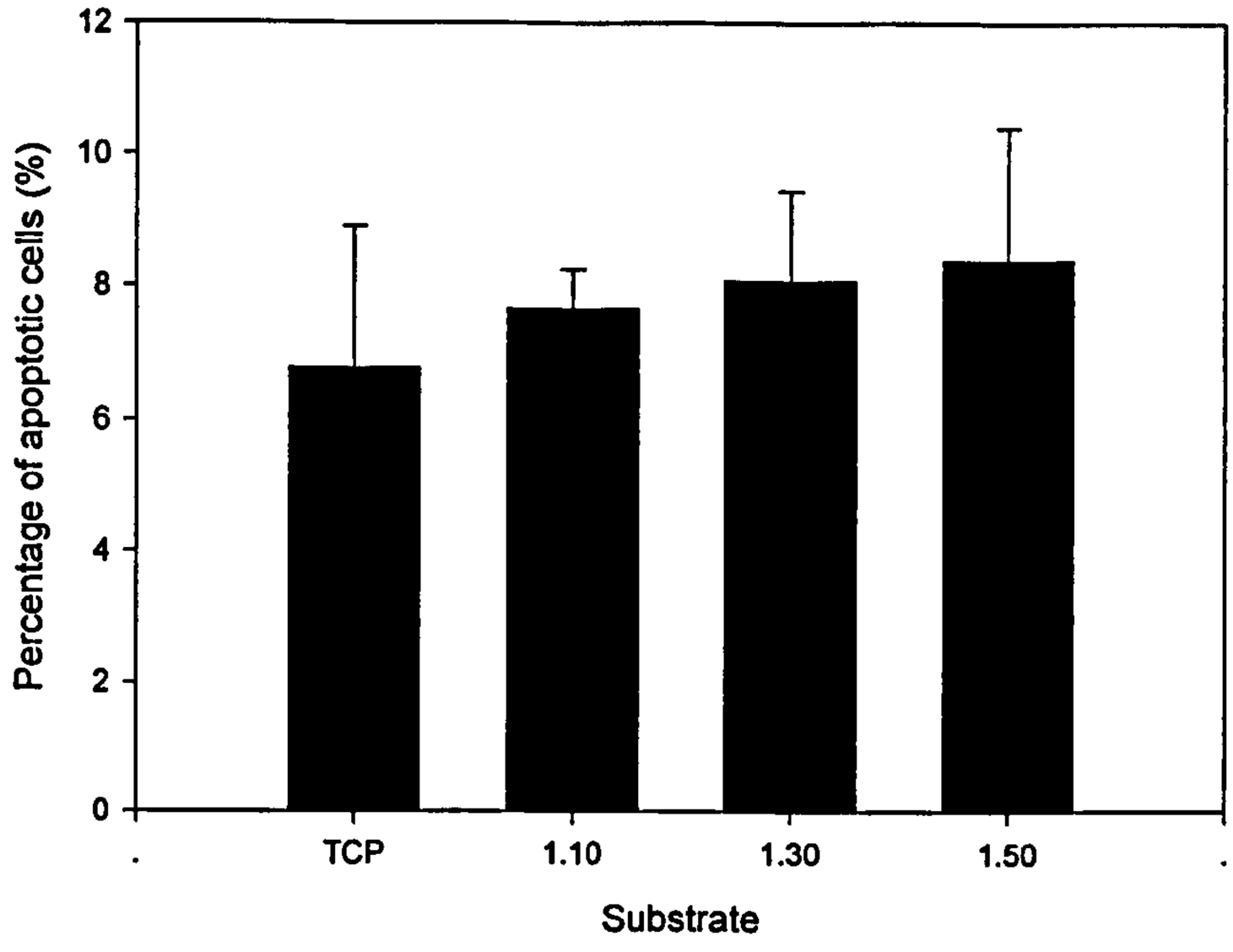


Figure 4-21: Effects of substrate stiffness on apoptosis levels in cultured MSC. 300 µl of primary MSC were plated out per 2-cm^2 well on PDMS substrates (1:10, 1:30 and 1:50) and coated TCP control in growth medium and cultured at 37 °C for 7 days. Apoptosis levels were ascertained by the positive staining for annexin V using FACS and data expressed as percent positive relative to the whole population (2000 events were read per sample). Data is presented as mean ± SD, representative of three individual experiments.

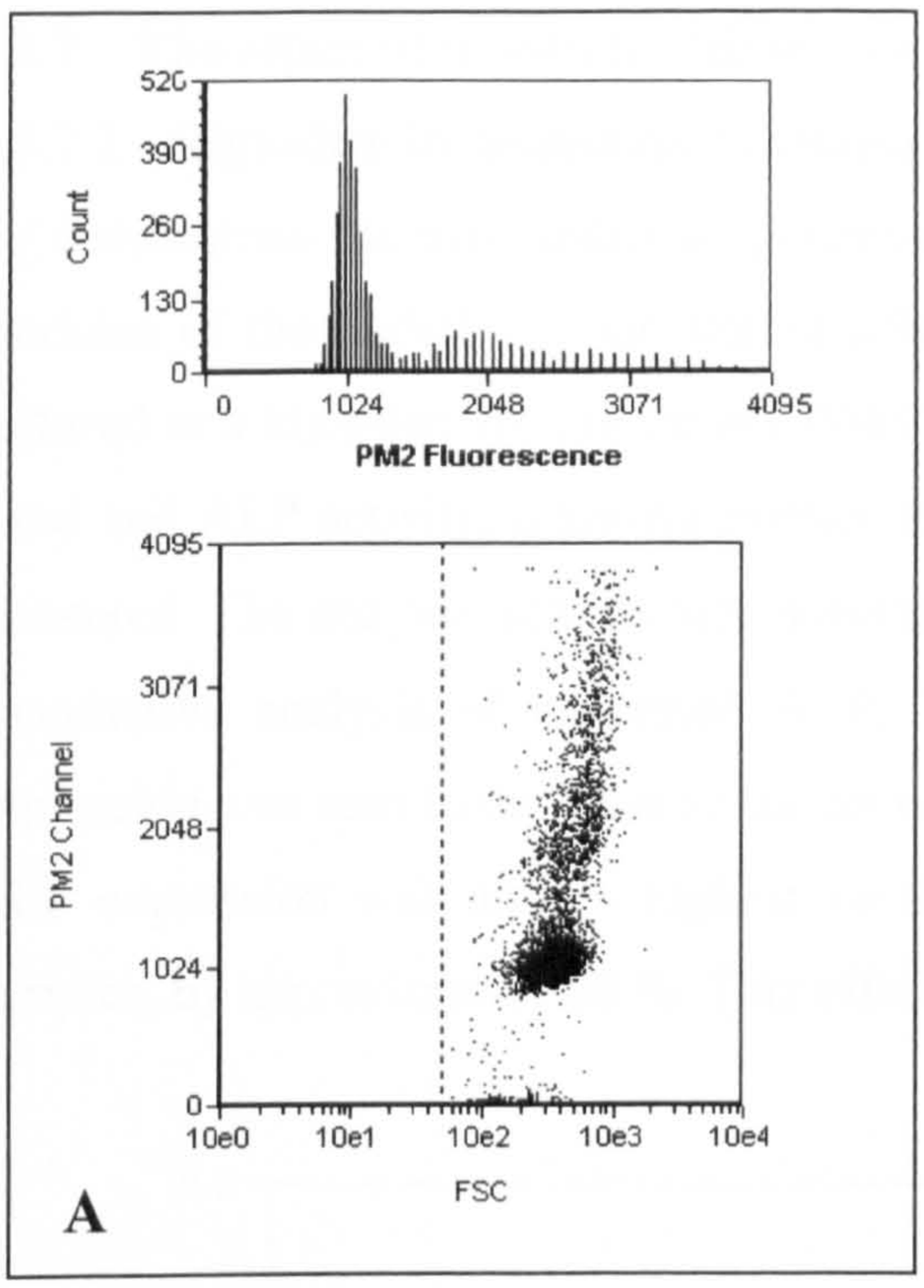
To determine if culture on the different substrates induced apoptosis, MSC were cultured for 7 days on an acrylic acid coated PDMS and an acrylic acid coated TCP control, before levels of annexin V were measured using a Guava PCA. Although there was a trend for levels of apoptosis to rise slightly as the stiffness of the substrate became softer. The differences seen were not statistically significant. Regardless of the substrate and relevant stiffness apoptosis was always below 10 % of the total cell population (**Figure 4-21**).

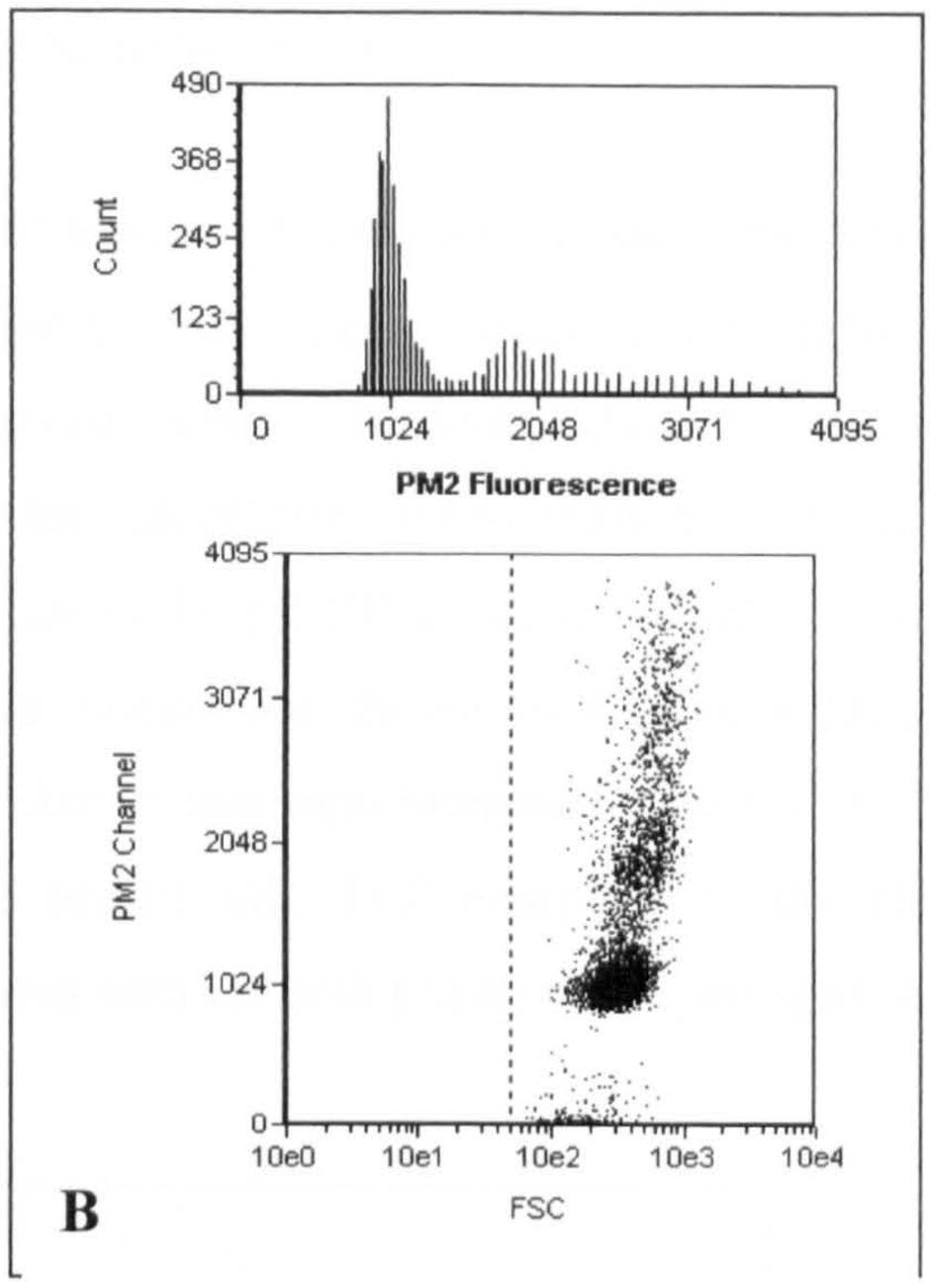
4.3.6.4 Effects of substrate stiffness on MSC DNA synthesis and cell cycle distribution

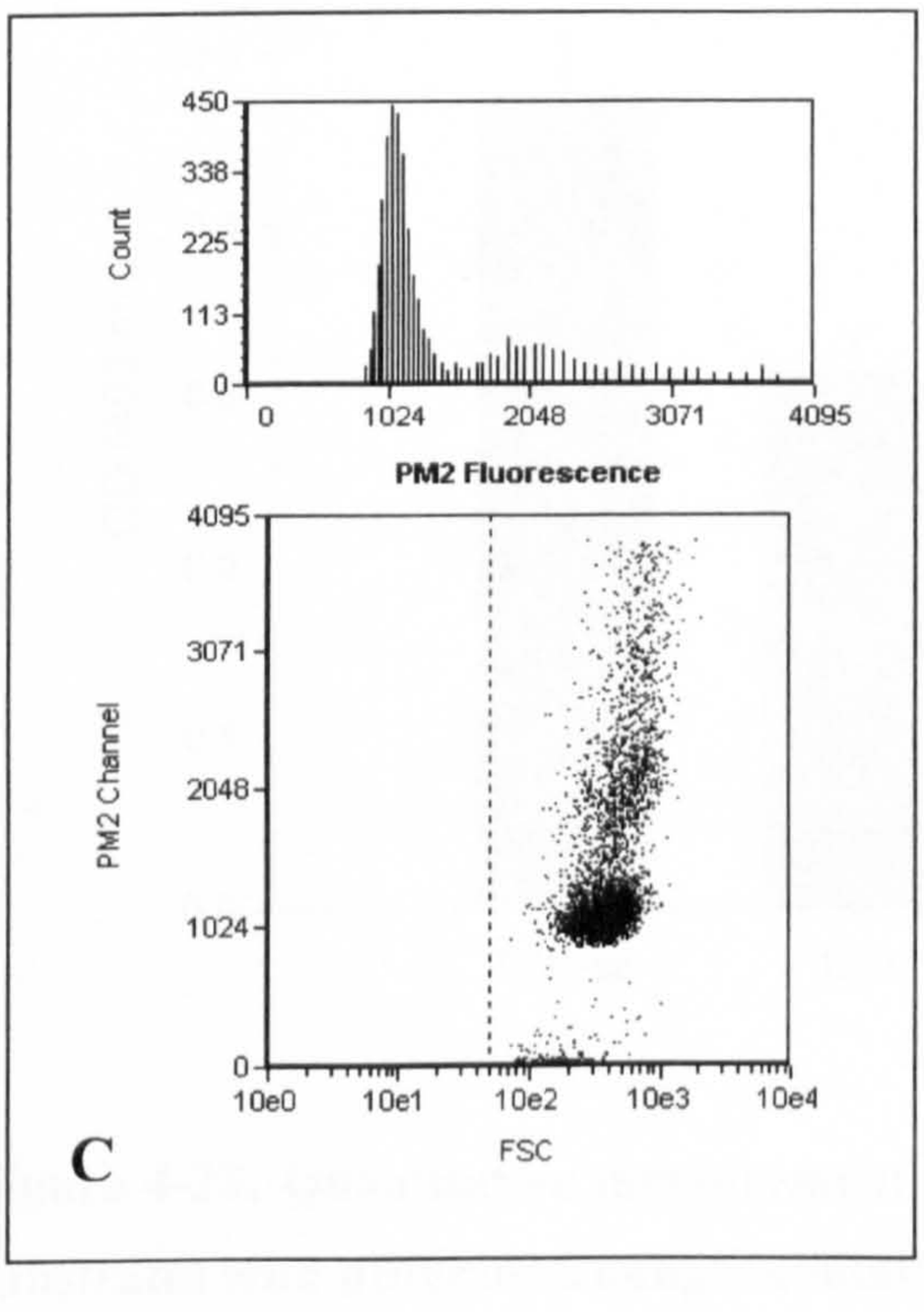
	Cell cycle analysis		
	G_0/G_1 (%)	S (%)	G_2/M (%)
TCP	83.0 ± 3.8	2.8 ± 0.6	14.2 ± 3.2
1:10	80.8 ± 1.9	4.9 ± 1.4	14.3 ± 2.3
1:30	78.4 ± 5.3	3.5 ± 0.8	18.1 ± 5.8
1:50	81.3 ± 1.6	4.5 ± 0.4	14.2 ± 1.3

Table 4-2: Cell cycle distribution analysis determined by FACS for cells cultured on a range of PDMS substrates (1:10, 1:30 and 1:50) and an acrylic acid coated TCP control. Data are shown as mean ± SD.

The effect of substrate stiffness on cell growth was further examined by DNA synthesis and cell cycle distribution analysis. As shown in **Table 4-2** there was no significant difference between the percentage of cells in any of the particular stages of the cell cycle at the time of the examination with an average of 81 % in G_0/G_1, 4 % in S and 15.2 % in G_2/M. This data is reflected in the images in **Figure 4-22.**







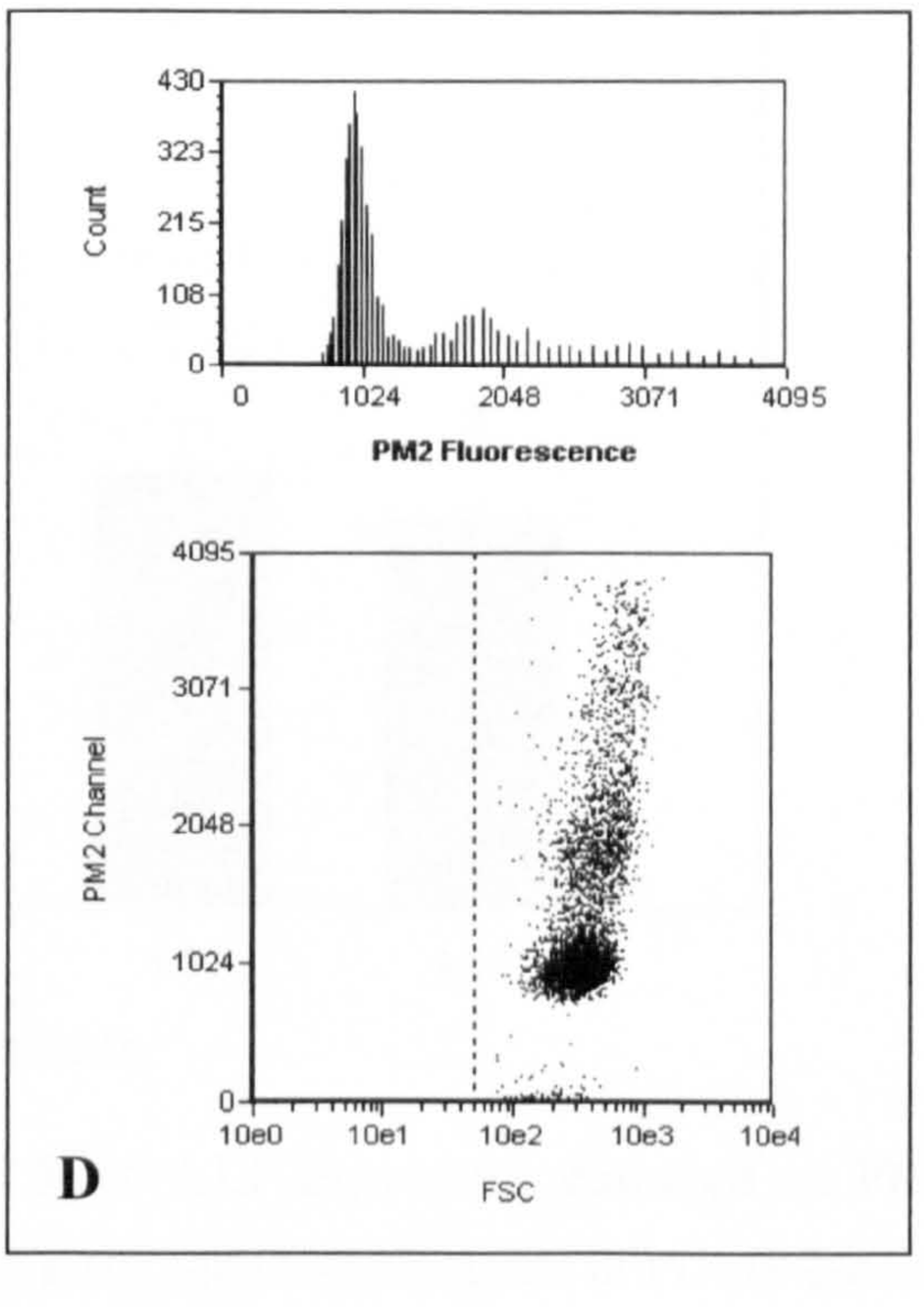


Figure 4-22: Representative PM2 channel flow cytometry data for the cell cycle analysis using the Guava PCA for cells after a 7 day culture period on (A) TCP and PDMS with varying crosslinker to base ratios (B) 1:10, (C) 1:30 and (D) 1:50.

4.3.7 The effect of substrate stiffness on MSC differentiation

4.3.7.1 High-density monolayer cultures

To demonstrate the differentiation potential of MSC on PDMS and to see if the Young's modulus of the underlying substratum affected the osteogenic lineage, 2 x10^4 cells were cultured as a high-density culture in PDMS coated 2-cm^2 wells. After 7 days, the cells were fixed and ALP activity, a known marker for the osteoblastic differentiation pathway, was measured. The enzyme activity was assessed using the p-NPP assay, which allows for the quantitative analysis of expressed ALP. The results are shown in **Figure 4-23**. ALP expression was seen to decrease as the crosslinker to base ratio increased from 1:10 to 1:50. ALP expression was always highest on the acrylic acid TCP compared to the PDMS samples, by approximately 80 %. This effect was seen for both primary and passaged cells.

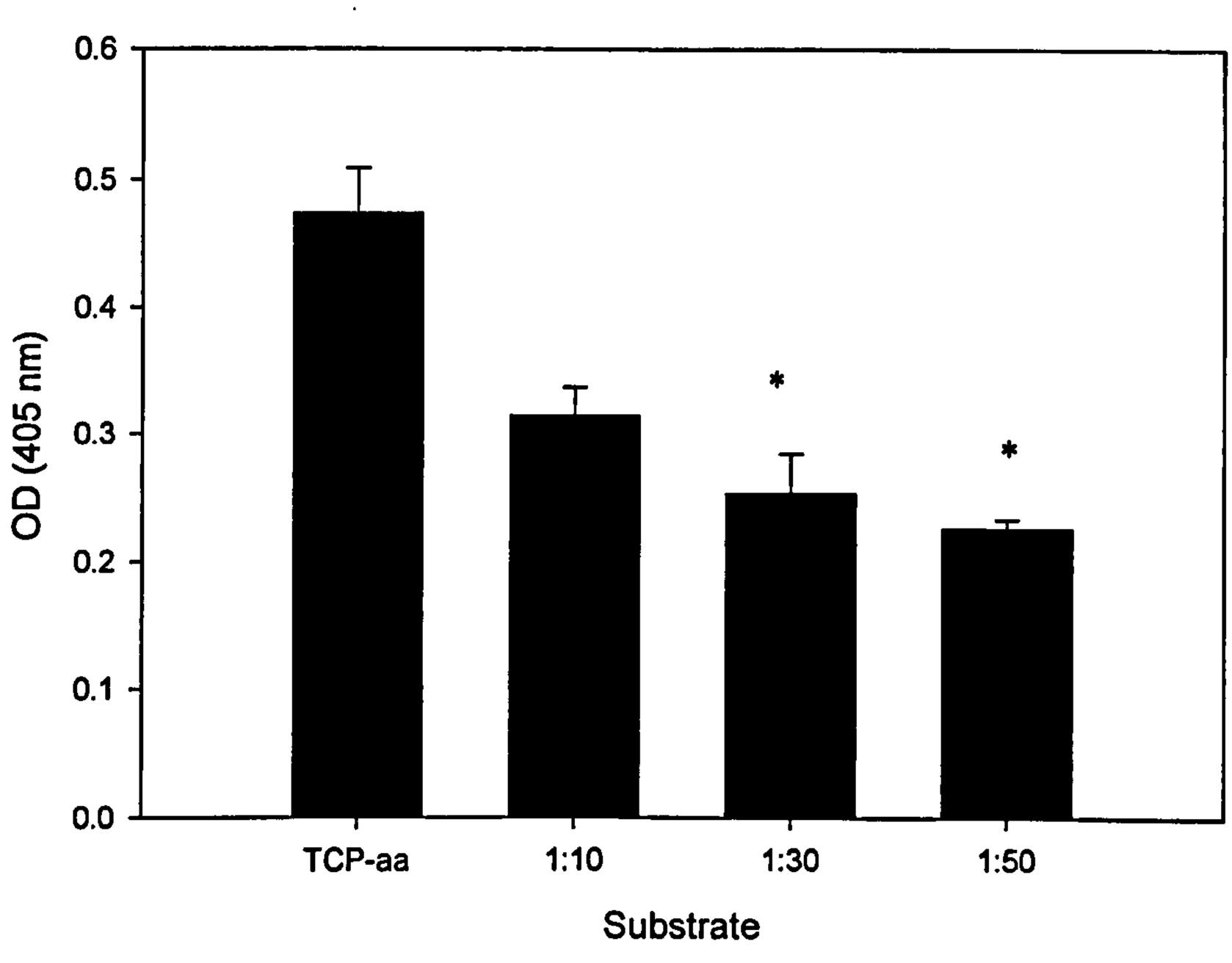


Figure 4-23: Quantitative measurement of MSC ALP expression cultured on PDMS substrates with different Young's moduli. 2 x 10^4 cells were cultured in PDMS coated 2-cm^2 wells in osteogenic medium. After 7 days the cells were fixed and the ALP expression measured colorimetrically using p-NPP (n=6). * denotes statistically significant difference from TCP control.

4.3.7.2 CFU-f

The CFU-f is the standard assay used for *in vitro* analysis and determination of mesenchymal tissue potential. 500 µl of primary bone marrow cells were seeded onto acrylic acid coated PDMS in 55-cm^2 dishes and cultured for 10 days in osteogenic medium. Histological staining for osteoblasts with naphthol phosphate/fast red showed the presence of osteoblasts on all the varying elastic substrates. As the degree of crosslinking increased, and hence the Young's modulus of the PDMS increased, the number of ALP colonies increased from 62 ± 29 on 1:50 to 88 ± 22 on 1:10 **(Figure 4-24)**.

Figure 4-25 shows an image of a representative plate for each of the ALP positive colonies cultured on the different substrates. Although there is an obvious trend the differences are not statistically significant. ALP positive average colony size was seen to be consistent for all of the PDMS crosslinker to base ratios with an average colony size of 0.08 ± .004 **(Figure 4-26)**. Colonies cultured on the TCP were significantly larger with an average size of 0.13, 63 % larger than those cultured on the PDMS substrates.

Total colony number, as determined by counting the colonies positive for the histological stain methylene blue, was seen to be slightly lower on the 1:30 and 1:50 PDMS substrates compared to the 1:10 and TCP control but not significantly so. On average there were 160 ± 17 colonies per dish. The average size of the colonies for the methylene blue stained colonies were larger when compared to the ALP positive colonies (**Figure 4-26**). However, the same trend was still observed when comparing the different substrates in that the PDMS colonies were smaller than the colonies cultured on TCP, typically by 65 %.

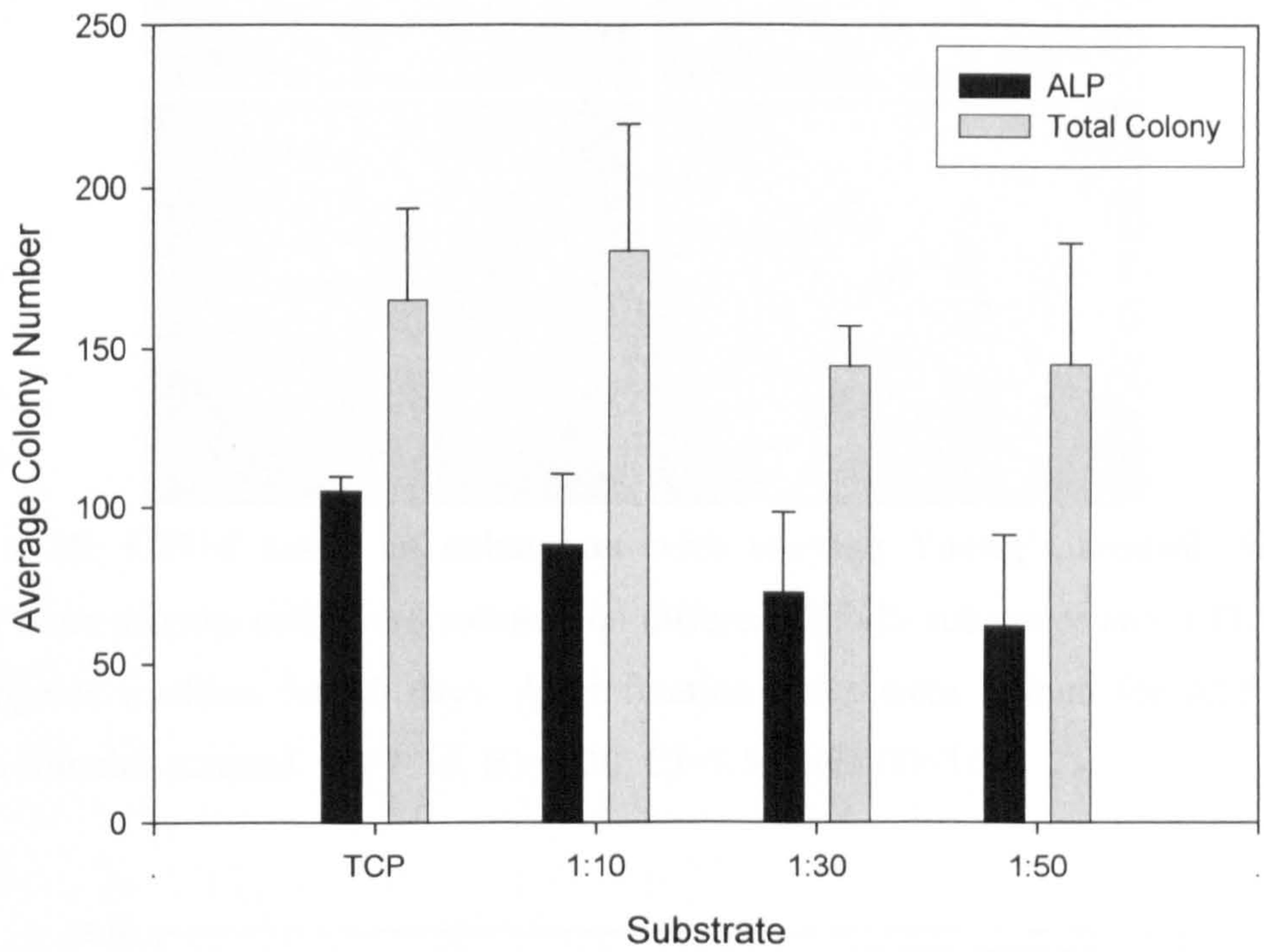


Figure 4-24: MSC were cultured on acrylic acid coated PDMS and TCP in 55 cm^2 plates and cultured for 10 days in osteogenic medium. After fixation, staining for alkaline phosphatase with naphthol phosphate/Fast red and for total colonies with methylene blue was performed. After each staining a digital image of each culture was acquired and the number and size of the colonies determined by image analysis. Data in this graph shows the mean number of ALP positive colonies and the mean total colony number for the different substrates ± SD (N=8).

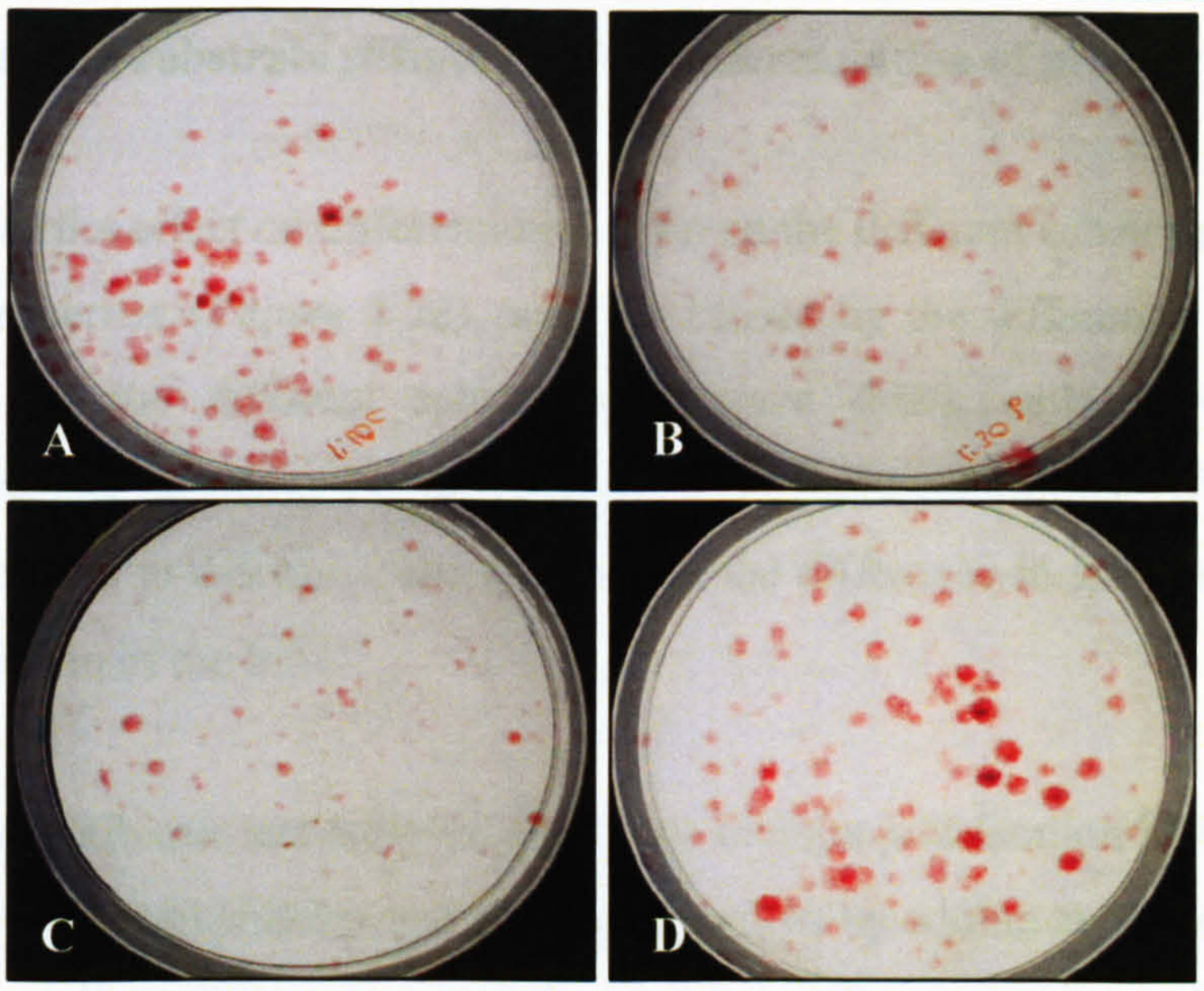


Figure 4-25: CFU-f assay on substrates with varying Young's moduli. 500 µl of primary bone marrow cells were cultured on different PDMS substrates and a TCP control in osteogenic medium for 10 days. After fixation, cells were stained for ALP and the positive colonies counted. A)=1:10, B)=1:30, C)=1:50, and D)=TCP.

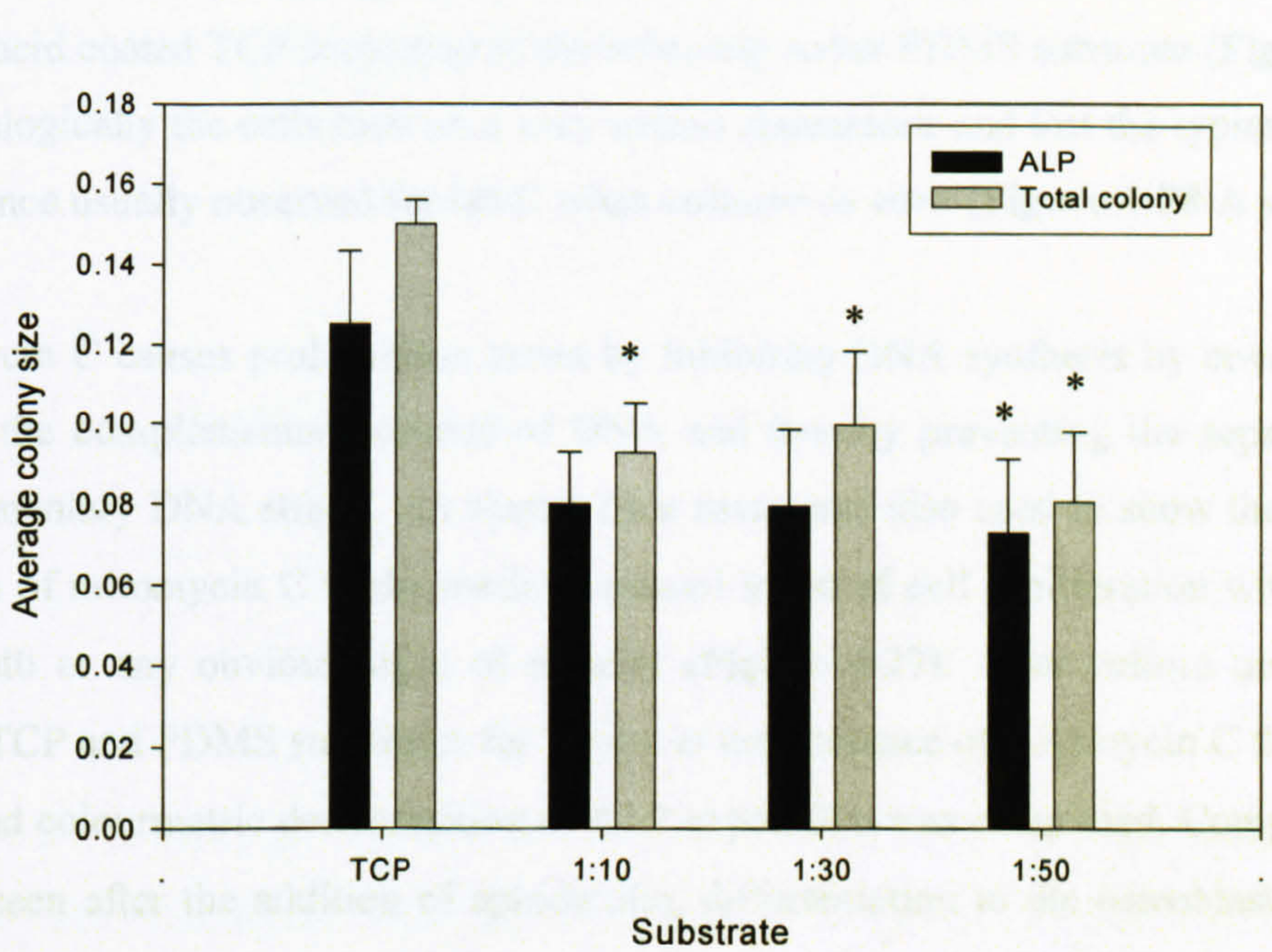


Figure 4-26: Average colony size per 55-cm^2 petri dish for both ALP positive cells and total colonies as determined by image analysis of the whole CFU-f culture. * denotes statistically significant difference from TCP control in corresponding group (N=3).

4.3.8 The effects of substrate stiffness on the differentiation of proliferation-arrested cells

To determine that the effect on differentiation seen on the different substrates, with varying mechanically properties **(Figure 4-23)**, was not caused by the difference in proliferation (also observed on the different substrates) **(Figure 4-19)**, cells were treated with proliferation-arresting agents and cultured under osteogenic conditions. After 7 days ALP levels were measured to determine the effects that the difference in substrate stiffness had on the differentiation of the MSC.

Aphidicolin is an antibiotic and antiviral agent that inhibits proliferation by inhibiting DNA polymerase. An alamar blue assay was used to show that by adding aphidicolin chronically to the medium, cell proliferation could be arrested without causing cell death or any obvious signs of toxicity **(Figure 4-27)**. After culture on acrylic acid coated TCP and PDMS substrates for 7 days, under osteogenic conditions in the presence of aphidicolin, the MSC were fixed and colorimetric determination of ALP expression was completed. Differentiation to the osteogenic lineage was seen to be significantly higher on the stiff acrylic acid coated TCP compared to the relatively softer PDMS substrate **(Figure 4-29-A)**. Morphologically the cells took on a very spread appearance and lost the typical fibroblastic appearance usually observed for MSC when cultured *in vitro* **(Figure 4-28-A and B)**.

Mitomycin C causes proliferation arrest by inhibiting DNA synthesis by covalently cross-linking the complementary strands of DNA and thereby preventing the separation of the complementary DNA strand. An alamar blue assay was also used to show that the chronic addition of mitomycin C to the medium caused arrest of cell proliferation without causing cell death or any obvious signs of toxicity **(Figure 4-27)**. After culture on acrylic acid coated TCP and PDMS substrates for 7 days in the presence of mitomycin C the MSC were fixed and colourmetric determination of ALP expression was completed. Comparable to the results seen after the addition of aphidicolin, differentiation to the osteoblastic phenotype (determined by staining for ALP) was significantly higher on the TCP substrate compared to the PDMS in proliferation-arrested cells **(Figure 4-29-B)**. Also the morphology of the cells was similar to that observed after the addition of aphidicolin in that the cells took on a more spread, and wiry appearance **(Figure 4-28-C)**.

Cytochalasin D is a cell permeable fungal toxin that acts as a potent inhibitor of actin polymerisation causing disruption to all actin microfilaments. Activation of the p53-dependent pathway causes arrest of the cell cycle at the G1-S transition. Addition of cytochalasin D to MSC caused the cells to have immediate loss of the actin stress fibre organisation **(Figure 4-28-D)**. Regardless to the change in cell shape and inhibition of the spread, fibroblastic morphology, ALP levels were seen to be significantly higher on the acrylic acid coated TCP compared to the PDMS substrate **(Figure 4-29-C)**.

Taken together these results from the proliferation arrested cells suggests that the increased differentiation seen on-high modulus matrices was not due to a reduction in cell proliferation.

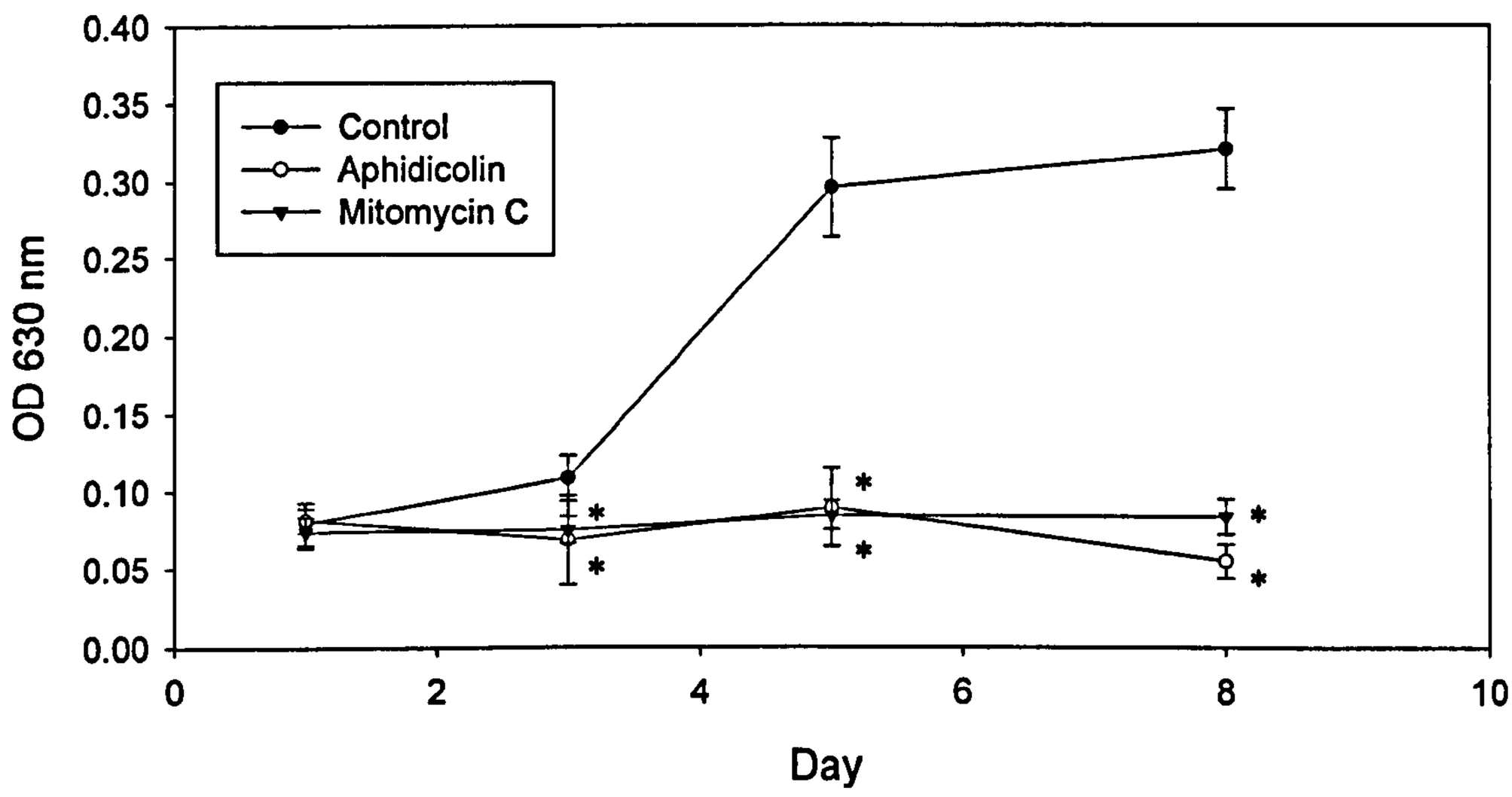


Figure 4-27: A MSC growth curve before and after the chronic addition of aphidicolin (2 μg/ml) and mitomycin C (50 μg/ml) over a 7-day period. The alamar blue assay was used to determine the proliferation rate of the cells. Untreated cells were used as a control. (N= 6). * denotes a statistically significant difference from the untreated control.

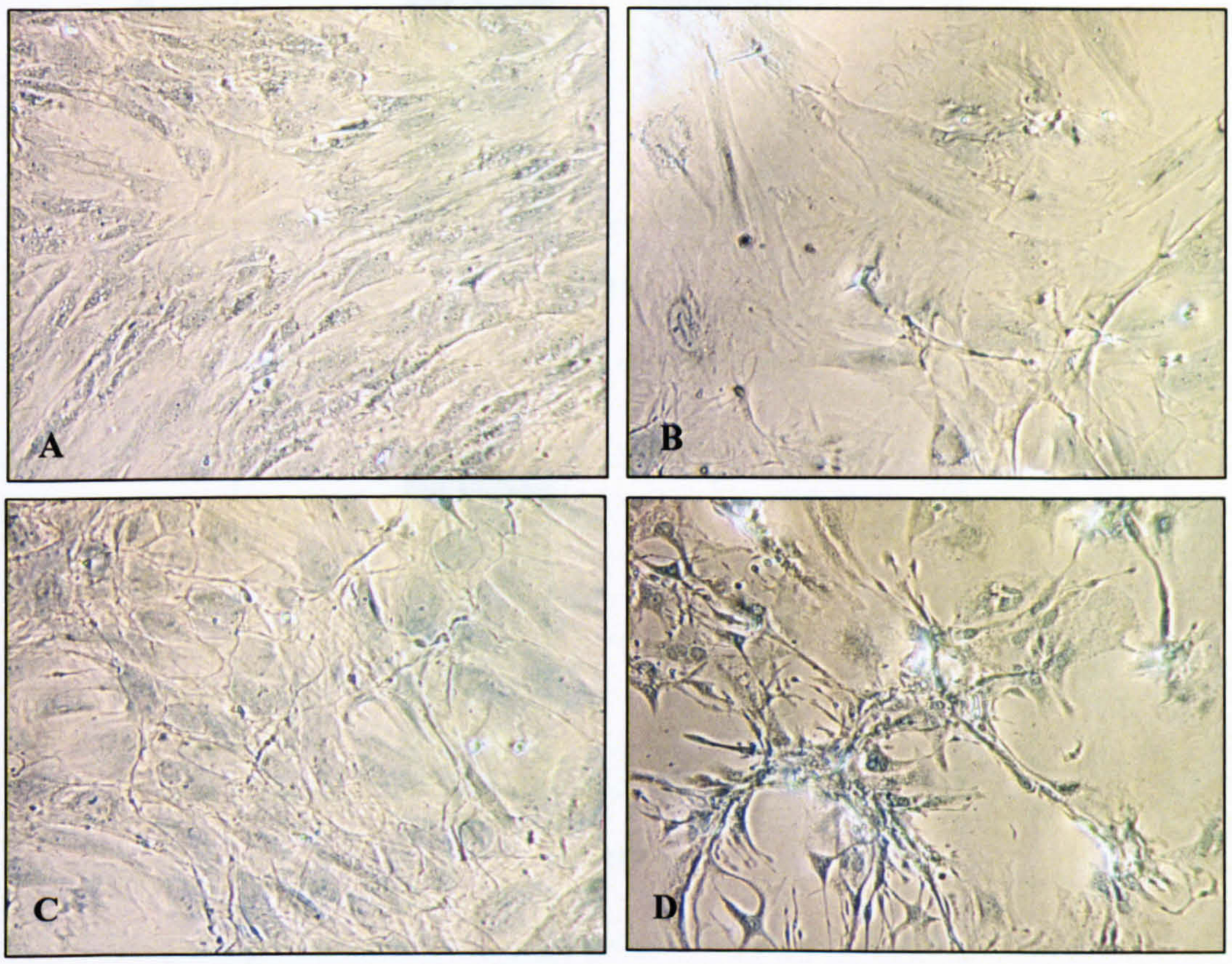


Figure 4-28: Brightfield images of methylene blue stained MSC cultured in osteogenic medium after 7 days with the addition of proliferation-arresting agents. (A) no agent (control) (B) aphidicolin (2 μg/ml) (C) mitomycin C (50 μg/ml) and (D) cytochalasin D (1 μg/ml) (Mag x 10).

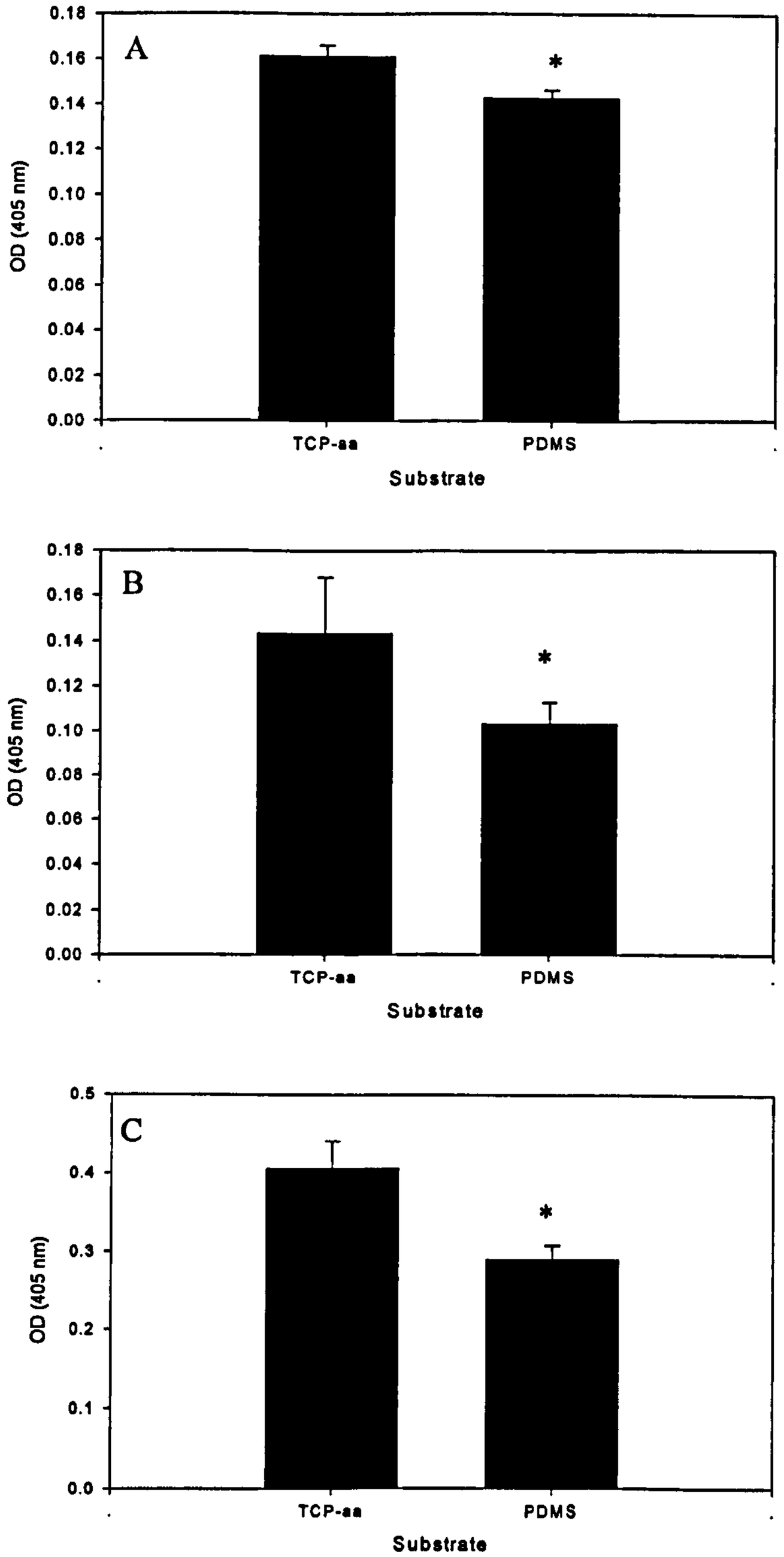


Figure 4-29: The effects of substrate stiffness on the ALP expression of MSC cultured on PDMS (1:30) and TCP in the presence of three proliferation arresting agents; (A) apidicolin (2 μg/ml), (B) mitomycin C (50 μg/ml) and (C) cytochalasin (1 μg/ml). 1 x 10^4 cells were cultured for 7 days at which point the cells were fixed and ALP expression quantitatively measured. Data presented represents three independent experiments ± SD (N=3). * denotes a significantly difference from the TCP control.

4.3.9 Effects of substrate stiffness on cytoskeletal impaired MSC.

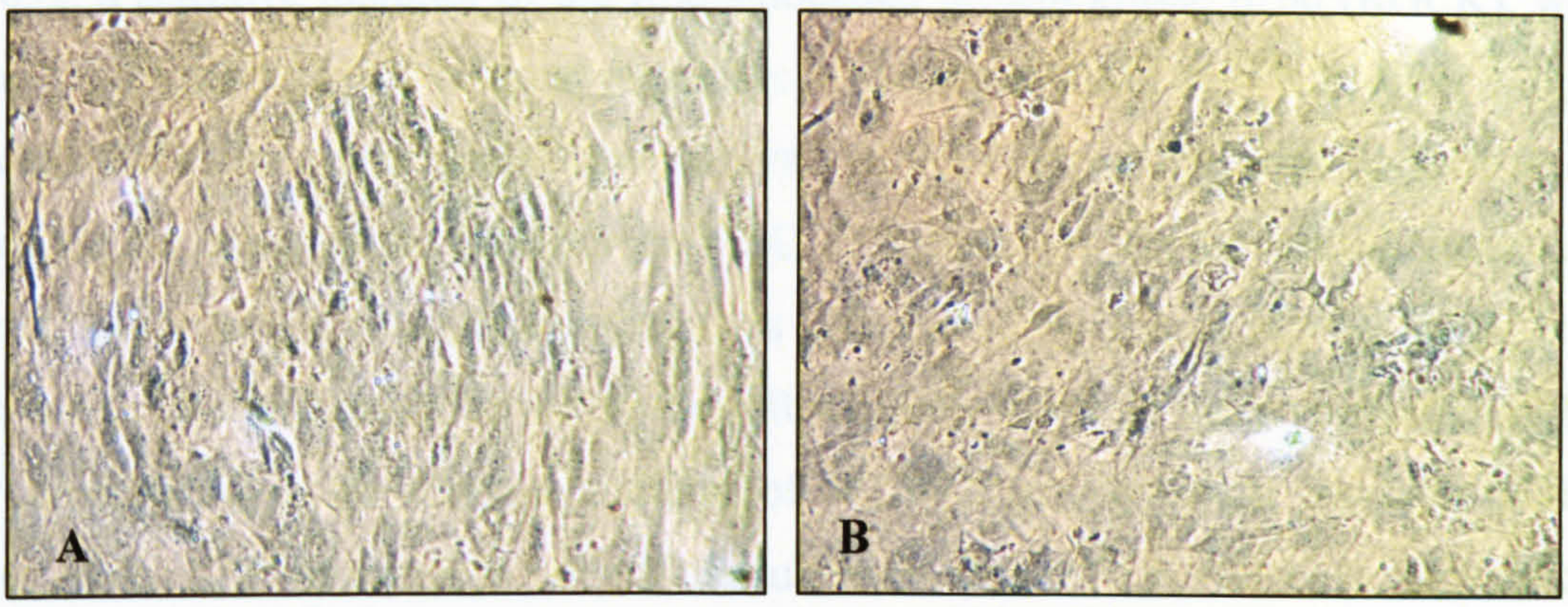


Figure 4-30: Brightfield images of methylene blue stained MSC. Cells were cultured in osteogenic medium on TCP with (A) or without (B) the daily addition of Y27632 (10 μM) for 7 days (mag x 10).

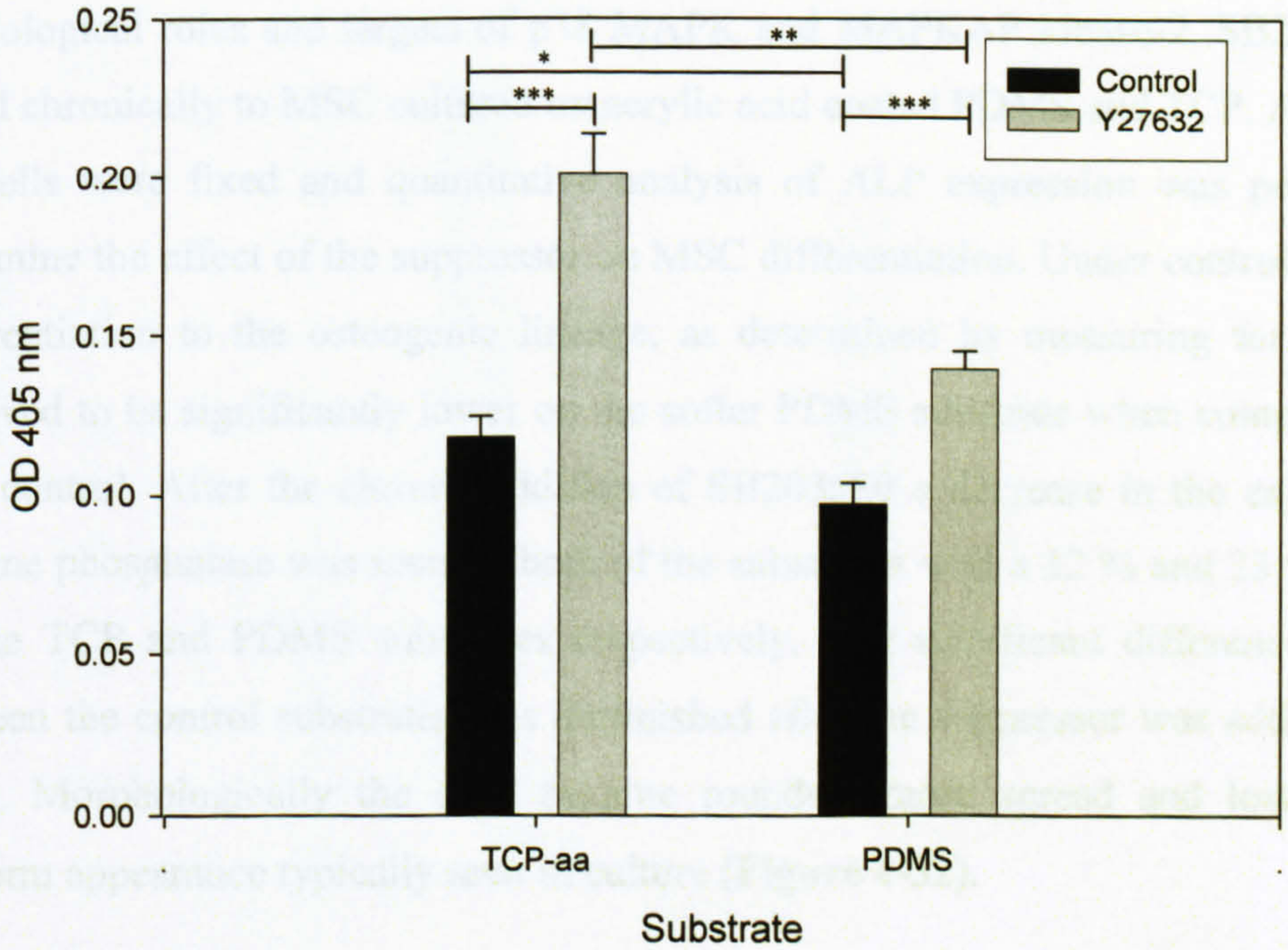


Figure 4-31: ALP expression of MSC cultured on PDMS and TCP with and without the addition of Y27632 (10 μM). 0.5 x 10^4 cells were cultured for 7 days at which point the cells were fixed and ALP expression quantitatively measured. Data presented represents three independent experiments ± SD (N=3). Statistical analysis was performed using a students T-test * = $p<0.017$, ** $p<0.002$, *** = $p<0.001$.

4.3.9.1 Y27632

Y27632, a selective inhibitor of the Rho-associated protein kinase p160 (ROCK), was added chronically to MSC cultured on acrylic acid coated PDMS and TCP. After 7 days the cells were fixed and quantitative analysis of ALP expression was performed to determine the effect of the inhibitor on MSC differentiation. Under control conditions differentiation to the osteogenic lineage, as determined by measuring for ALP, was observed to be significantly lower on the softer PDMS substrate when compared to the TCP control. After the chronic addition of Y27632 an significant increase in the expression of ALP was seen on both of the substrates with a 70 % and 43 % increase on the TCP and PDMS substrates respectively **(Figure 4-31)**. Morphologically the confluent cells remained spread and although the cells were slightly less fusiform, the morphology was comparable to untreated MSC **(Figure 4-30).**

SB203580 is a pyridinyl imidazole that suppresses the activation of MAPKAP kinase-2. It does not inhibit JNK or p42 MAP kinase and, therefore, is useful for studying the physiological roles and targets of p38 MAPK and MAPKAP kinase-2. SB203580 was added chronically to MSC cultured on acrylic acid coated PDMS and TCP. After 7 days the cells were fixed and quantitative analysis of ALP expression was performed to determine the effect of the suppressor on MSC differentiation. Under control conditions differentiation to the osteogenic lineage, as determined by measuring for ALP, was observed to be significantly lower on the softer PDMS substrate when compared to the TCP control. After the chronic addition of SB203580 a decrease in the expression of alkaline phosphatase was seen on both of the substrates with a 32 % and 23 % decrease on the TCP and PDMS substrates respectively. The significant difference observed between the control substrates was diminished after the suppressor was added **(Figure 4-33)**. Morphologically the cells became rounded, more spread and lost the usual fusiform appearance typically seen in culture **(Figure 4-32).**

4.3.9.2 SB203580

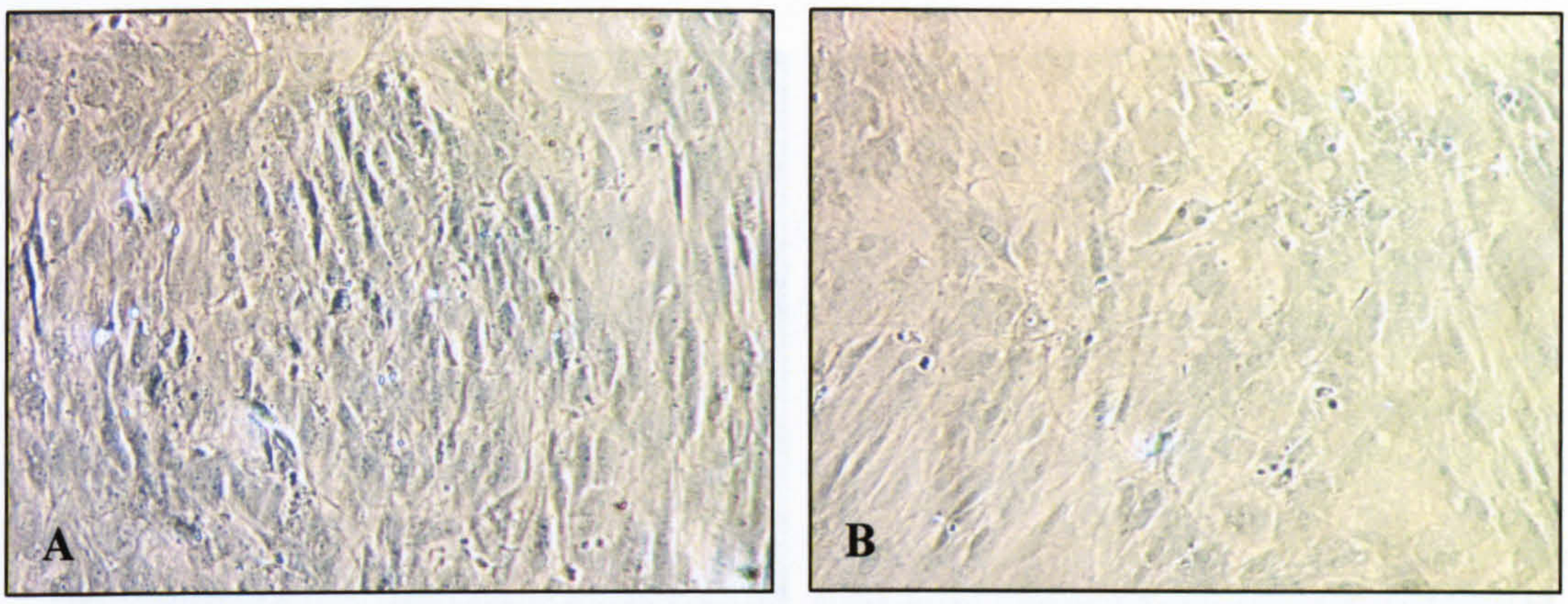


Figure 4-32: Phase contrast images of MSC on TCP cultured in osteogenic medium A) with or B) without the addition of SB203580 (10 μM) (mag x 10).

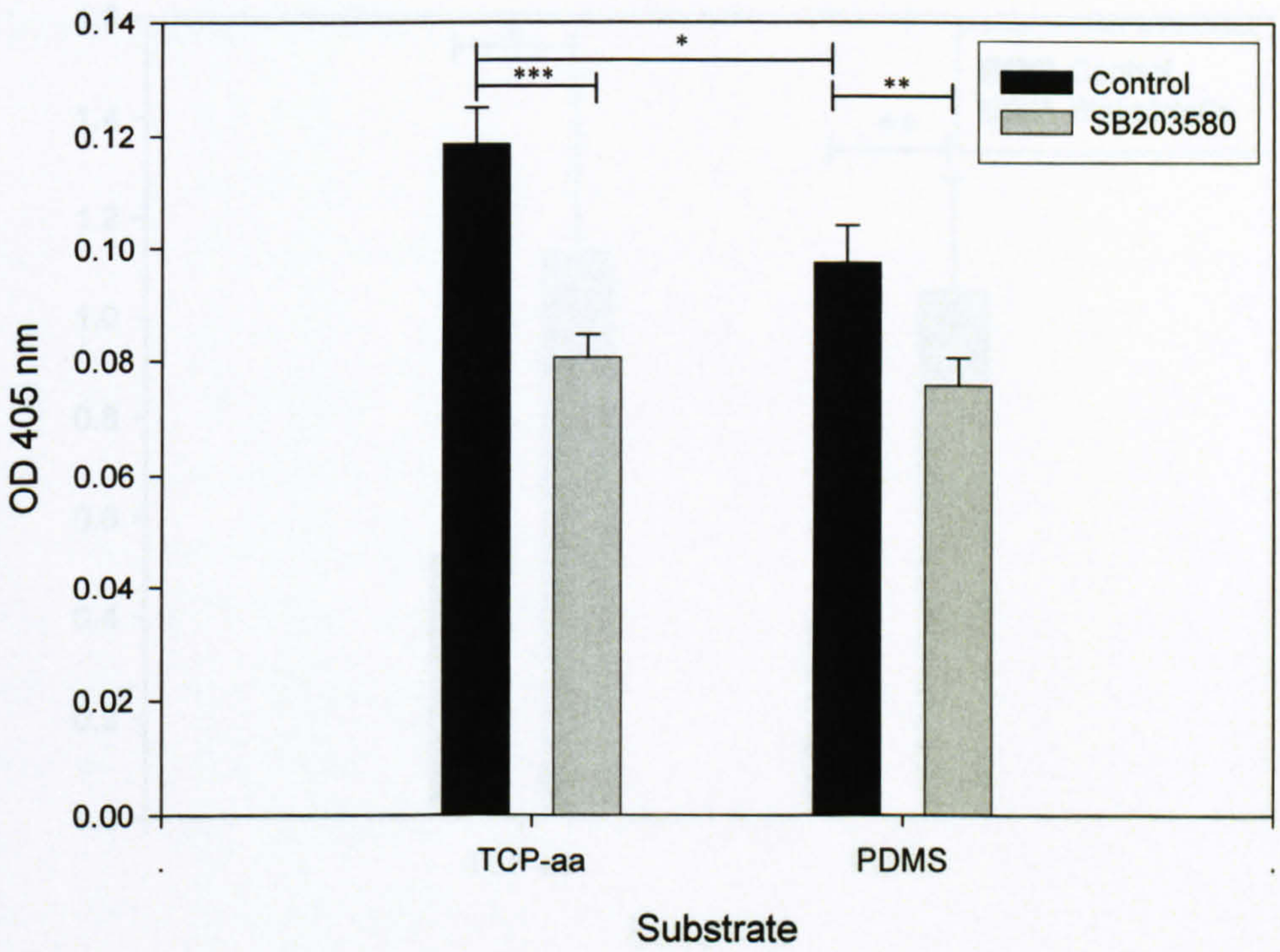


Figure 4-33: ALP expression of MSC cultured on PDMS and TCP with and without the addition of SB203580 (10 μM). 0.5 x 10^4 cells were cultured for 7 days at which point the cells were fixed and ALP expression quantitatively measured. Data presented represents three independent experiments ± SD (N=3). Statistical analysis was performed using a students T-test * = $p<0.017$, ** $p<0.01$, *** = $p<0.001$.

4.3.9.3 Blebbistatin

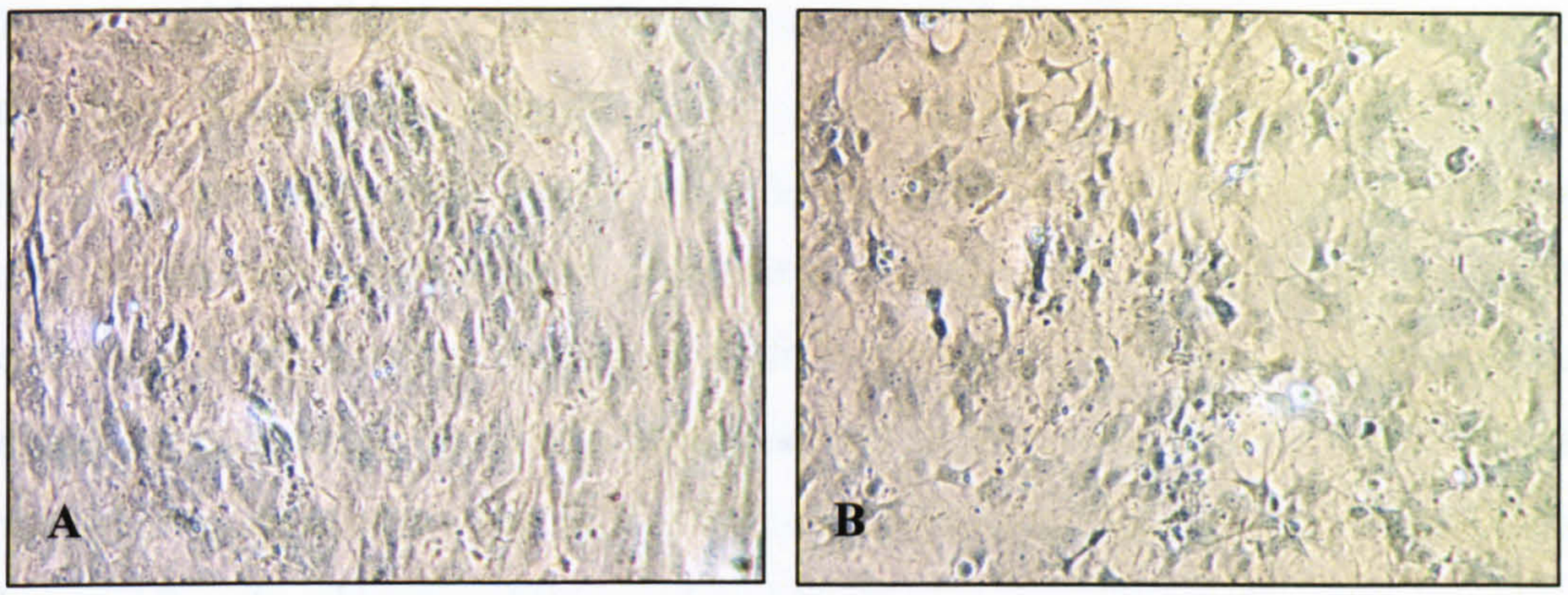


Figure 4-34: Phase contrast images of MSC on TCP cultured in osteogenic medium with (A) or without (B) the addition of Blebbistatin (50 μM) (mag x 10).

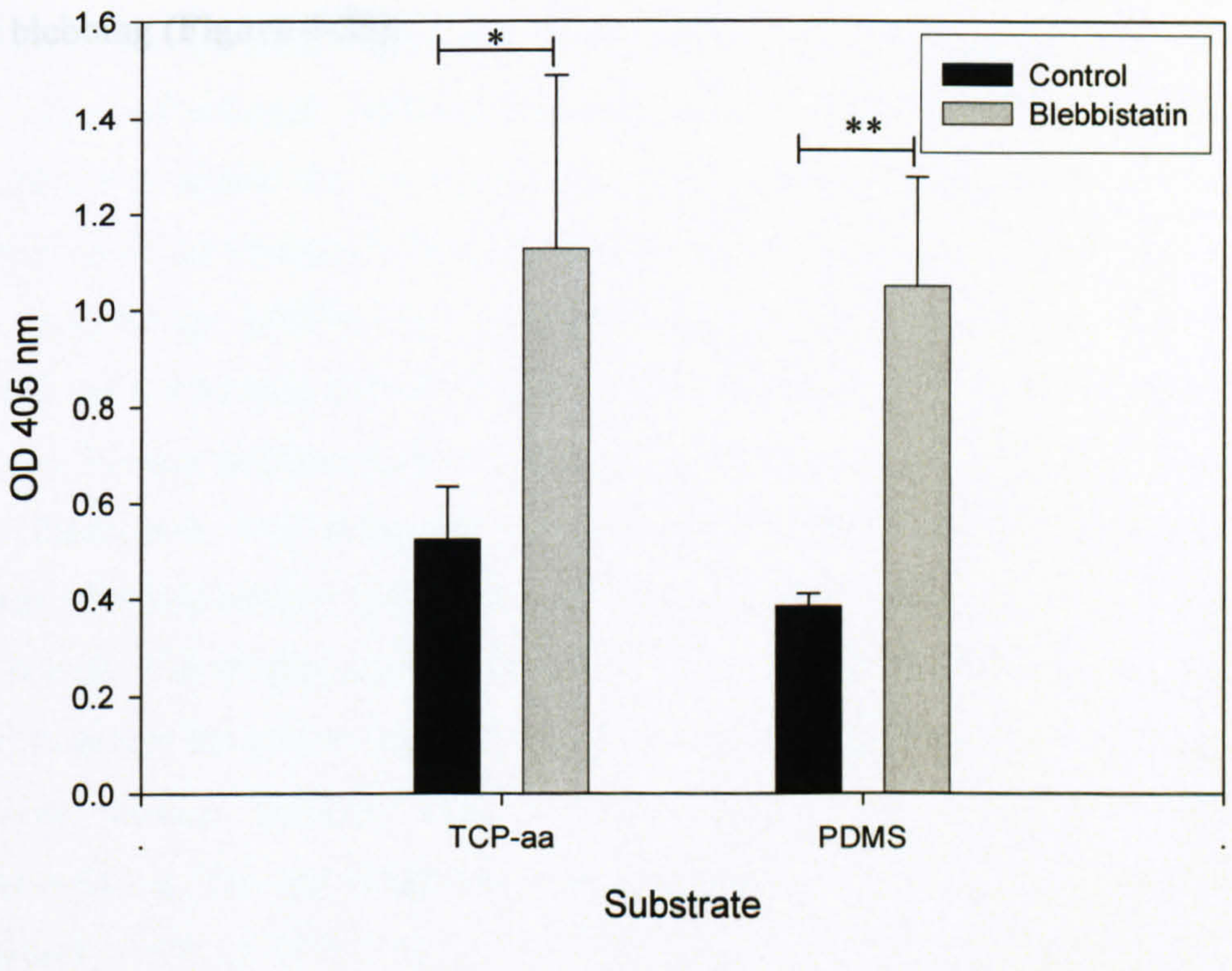


Figure 4-35: ALP expression of MSC cultured on PDMS and TCP with and without the addition of blebbistatin (50 μM). 0.5 x 10^4 cells were cultured for 7 days at which point the cells were fixed and ALP expression quantitatively measured. Data presented represents three independent experiments ± SD (N=3). Statistical analysis was performed using a students T-test * = $p<0.05$, ** $p<0.007$.

Blebbistatin is a cell permeable, specific inhibitor of non-muscle myosin II that causes inhibition of cell migration, invasiveness and impairment of cell spreading. It is therefore a useful inhibitor to use for the study of the role of myosin II in MSC differentiation. Blebbistatin was added chronically to MSC cultured on acrylic acid coated PDMS and TCP. After 7 days the cells were fixed and quantitative analysis of ALP expression was performed to determine the effect of the inhibitor on MSC differentiation. Under control conditions differentiation to the osteogenic lineage, as determined by measuring for ALP, was observed to be lower on the softer PDMS substrate when compared to the TCP control however this data was not statistically significant. After the chronic addition of blebbistatin an increase in the expression of alkaline phosphatase was seen on both of the substrates with a 115 % and 172 % increase on the TCP and PDMS substrates respectively. The difference in ALP expression observed under control conditions was also diminished after the suppressor was added **(Figure 4-34).** Morphologically the cells became rounded with evidence of cell blebbing **(Figure 4-35).**

4.4 Discussion

Silicone is already used extensively as a biomaterial in a number of applications including contact lenses (Hill and Schoessler, 1967), catheters (Haid et al., 1988), heart valves (Mullison, 1964), artificial limbs (Marks and Michael, 2001) and breast implants (Weiner et al., 1974). Its good optical clarity, low permeability to water and its thermal stability contribute to making it a highly desirable biomaterial (Ai et al., 2002). These properties along with the large range of Young's moduli that can easily be created have led to its use in the study of elastic substrates on cell behaviour. Artificial substrates offer a number of advantages over natural substrates and remove the unpredictable associated with the use of protein substrates. For the study of substrate stiffness on cellular behaviour it is paramount that as many of the variables are controlled as possible, to limit the factors causing any effect and thereby allowing true extrapolation of results.

We have demonstrated that PDMS can be used as an artificial substrate for the study of the effects of substrate stiffness on MSC growth. Once cast, mechanical testing was completed to ensure that by simply altering the degree of crosslinking that the elastic properties of the material were indeed being altered. Using both cell attachment assays and morphology profiles we determined that uncoated and protein adsorbed coated PDMS did not support the initial attachment and morphology of MSC typically seen in culture. Plasma polymerisation, a technique commonly used on materials to enhance cell attachment, was employed and proved to enhance the attachment of MSC in culture. Surface science techniques enabled us to characterise the coating and to study the effects of time upon coating integrity. A number of cell-based techniques were then used to assess the growth of the cells on the different substrates and to determine as to whether surface stiffness does actually have an effect on MSC growth and differentiation. The use of inhibitors were then used to elucidate a possible mechanism of action.

We confirmed that by simply changing the ratio of oligomer to crosslinker you could easily change the elastic properties of the resultant polymer with attainable Young's modulus ranging from 66 kPa ± 9.08kPa to 1.1 MPa ± 0.1 Mpa (**Figure 4-1**). These moduli, attained using bulk tensile testing, are comparable to published data (Brown et al., 2005), however, the modulus obtained is for the bulk of the material and therefore

averages out the moduli for the material and is not accurate enough to pick up any heterogeneities on the micron scale. The time frame in which the tension testing is completed will also make a difference and therefore should be recorded to enable comparisons between data.

PDMS offers a material that is convenient for the study of the elastic properties of the underlying substrate on stem cell growth and differentiation. But by changing the degree of crosslinking 'what other properties are changing?' Fabrication with less curing agent leaves more vinyl-terminated dimethylsiloxane units uncross-linked and consequently increases the amount of platinum catalyst and silica filler (dimethylvinylated and trimethylated silica) in the polymer, thereby effecting the number of free and mobile polymer chains (Lee et al., 2004). In the present study, analysis of the surface by XPS detected no evidence of platinum within the system; however, any silica filler would have been indistinguishable within the spectra and its presence could therefore not be ruled out. Changes in the degree of free and mobile polymer chains alone could also be having an effect on the cells either directly or via a change in the roughness of the material. Variations in the nano-topography of surfaces are known to influence cellular behaviour (Dalby et al., 2004). Micro-rough surfaces can inhibit proliferation whilst enhancing osteogenic differentiation (Boyan et al., 2003). Unfortunately, employing the technique atomic force microscopy we were unable to get mean roughness recordings for the PDMS substrates due to the 'sticky' nature of the polymer. The topography of the surface is a feature that should also be considered when plasma coatings are applied. Plasma polymers are generally conformal Yasuda, (1985), so even after a coating is applied the surface roughness is controlled by the underlying substrate and therefore surface roughness data should be obtained.

In the case of the uncoated PDMS substrates, cell attachment in medium with 10 % serum was significantly lower compared to the standard TCP. Interestingly, we report that the initial attachment in serum free medium is significantly higher than that observed in medium with 10 % serum on the PDMS substrates but not the TCP control. On the PDMS, it is possible that serum rich conditions promotes the adsorption of albumin, which impedes cells attachment (McFarland et al., 1999), but under serum free conditions, where no albumin is added, cells have to lay down their own ECM to enable attachment. In addition, the hydrophobic nature of the surface causes proteins that are

adsorbed to the surface to be in a conformation that limits cell adhesion, with integrins not available for binding. Regardless to this difference in the numbers of cell attaching, morphologically the cells remained round with no spreading seen on all the uncoated PDMS substrates in both the serum free and serum rich conditions. The cells also remained rounded on the TCP in serum free conditions compared to a typical fibroblastic morphology seen on the TCP in serum rich conditions consistent with the knowledge that serum is needed for the attachment and spreading of adherent cells in culture (**Figure 4-2, Figure 4-3**).

Fibronectin, one of the two main adhesion proteins that adsorb to biomaterials, is commonly used to enhance cell adhesion (Cunningham et al., 2002). However pre-adsorption of a fibronectin layer, did not improve cell attachment in serum rich conditions on the PDMS substrates (although it did on the TCP control) and again morphologically the cells were unable to spread and take on the fusiform appearance typically seen in culture (**Figure 4-5, Figure 4-6**). As fibronectin improves attachment on TCP and not PDMS it strongly suggests that physiochemical interactions of the protein with the PDMS substrate does alter the presentation of binding sites due to the hydrophobic nature of the PDMS (Steele et al., 1994). Similar to the uncoated PDMS, cell attachment was observed to be higher under serum free conditions with pre-adsorbed fibronectin only affecting the TCP control. Proving, thereby, that fibronectin was present in the system. It is also feasible that the fibronectin is not adsorbing to the PDMS. This result has previously been reported (Cunningham et al., 2002) and is why the Flexicell® system (Flexcell International Corporation, USA) an automated instrument that stretches cells in a 2-D monolayer now uses silicone covalently coated with ECM (BIOflex, Flexcell International Corporation, USA).

Brown et al., (2005) have demonstrated that under serum free conditions, cell attachment onto PDMS substrates is correlated to substrate stiffness with VSMC attaching preferentially to stiffer substrates. However, either the pre-adsorption of fibronectin or the addition of serum removed the effect seen. In the work presented here, no stiffness related change in the number of cells attaching was seen which suggests that the dissimilarity between the studies is due to the difference in cell type and or surface coating.

Due to the inefficiency of a pre-adsorbed protein layer to enhance cell adhesion it was decided that a surface coating was needed to effectively lower the contact angle of the PDMS and render the surface hydrophilic. Although TCP is the standard *in vitro* culture substratum and for the purpose of this study a control substrate, our aim was not to better TCP but to successfully culture MSC on PDMS with a range of mechanical properties. However, comparison studies between acrylic acid coated and not-coated TCP demonstrated no significant difference in the attachment or growth of MSC **(Figure 4-7 and Figure 4-8).** Furthermore, previous studies have demonstrated the effectiveness of acrylic acid coated substrates for cell culture purposes (Daw et al., 1998). It must also be noted that the use of an artificial chemical layer as a surface coating to enhance cell attachment means that a controlled chemical environment is ensured. Most other studies using artificial substrates as a cell culture substratum don't address the issue that subtle changes in the surface chemistry may affect cell adhesion, or cause different proteins to be adsorbed from the serum. It has been shown that cell proliferation and differentiation can be directed alone through conformational changes in extracellular matrix proteins (Garcia et al., 1999). This is why chemical characterisation of the substrates was completed before extrapolation of cell behaviour data.

XPS data for the uncoated samples showed the presence of carbon, oxygen and silicon on the substrates regardless of the crosslinking density. Atomic elemental composition was on average 44 % carbon, 27 % oxygen and 29 % silicon. These values are slightly different than the expected theoretical values of 50 %, 25 % and 25 % for carbon, oxygen and silicon (Briggs and Seah., 1990) and is possibly due to the formulation of the PDMS.

Coating with acrylic acid significantly increased the amount of carbon and oxygen present on all of the surfaces and attenuated the amount of silicon seen. However, on the 1:50 higher levels of silicon were seen suggesting that the coating is not sufficiently covering the PDMS. However, there is no evidence of a rising background under the Si peaks, suggesting that either the monomer/polymer is being incorporated into the polymer or that the PDMS is mobile and is migrating over the plasma polymer film. The first theory fits with the observation that the 1:50 PDMS substrate turned cloudy during the plasma polymerisation process and that the monomer/polymer is mixing with

the PDMS. The narrow scan spectra revealed the presence of an acid shoulder on all of the substrates, however there was a peak shift and the acid functionality decreased as the crosslinker ratio increased. Regardless of the coating, the contact angle measurement was reduced and would suggest that is why there was no difference in the number of cells attaching compared to the other two substrates.

For acrylic acid coated PDMS to be used as a cell culture substratum, storage of the polymers with the coating is essential to enable reproducibility of experiments. Acrylic acid coated PDMS stored in PBS at 37 °C for 14 days showed no evidence of an acrylic acid coating after incubation. However, when stored at 4 °C there was evidence of a coating on the 1:10 and 1:30 (**Figure 4-14**). The depletion in the coating is probably due to hydrophobic recovery caused by reptation, a phenomenon seen when PDMS is oxygen treated to make it hydrophilic with reorientation in air rapidly observed, a problem not encountered if the sample is kept in water (Lee et al., 2004). Despite the loss of the coating overtime there was no evidence of cells lifting off suggesting that once they had laid down their own ECM the integrity of the coating is not a problem.

Contact angle measurements showed that the addition of the acrylic acid layer to all of the PDMS substrates rendered them more hydrophilic (**Figure 4-16**) enabling protein adsorption and subsequent cell attachment. Hydrophilic surfaces favour the retention of native protein conformation which is often lost on the irreversible binding to hydrophobic surfaces (Underwood et al., 1993). The effect of reducing the surface contact angle from an average of 75° ± 5° to 43° ± 3° could be seen by the change in the extent of cell attachment (**Figure 4-17**). No difference in the contact angle measurements were seen between the uncoated PDMS substrates (regardless of crosslinker to base ratio) but once the acrylic acid coating was added the 1:10 substrate had a slightly lower contact angle compared to the 1:30 and 1:50 and possibly suggests that the coating was better on the 1:10. There is no evidence to support the fact that contact angles alone can alter cell attachment but reflects a difference in the surface chemistry that can subsequently alter protein adsorption. The contact angle data presented here for the uncoated samples is lower compared to other published readings (Brown et al., 2005) but it must be taken into consideration that contact angles are notoriously user independent and an error of ± 4 is always expected (Ratner et al., 2000).

As previously stressed, to accurately study the effects of substrate stiffness on cellular behaviour all other variables must be recognised, considered and controlled. As well as lowering the contact angle, (enabling protein adsorption with native conformation and thereby promoting cellular attachment) using a plasma polymerised coating means you can look at the exact chemistry of the substrate and enables tighter control compared to adsorption of ECM proteins alone. It also allows the stability of the coating to be assessed over time and evaluation of the coating's integrity and suitability for long-term culture.

As previously stated, bulk mechanical testing data gives information regarding the material as a whole and doesn't allow for small more localised heterogeneities on a scale relative to the cell. It must also be taken into consideration that the addition of the acrylic acid layer could change the mechanical properties of the polymer either directly or during the plasma polymerisation process, when the polymer was placed under vacuum conditions. The more localised mechanical testing data showed that there was no significant difference between samples before or after the addition of the acrylic acid coating (**Figure 4-16**).

Quantative cell attachment data revealed that after treatment with acrylic acid, cell attachment on PDMS was improved significantly compared to uncoated samples and matched the attachment levels seen in TCP samples for both serum rich (10 %) and serum free conditions (**Figure 4-17**). Furthermore, there was no significant difference in attachment between the serum free and serum rich conditions and suggests that the phenomenon of more cells attaching under serum free conditions was artificial as the substrates did not support cell growth. Previous research has demonstrated that fibronectin (as one of the two most important adhesion proteins) has a strong affinity for COOH- surfaces and is therefore able to promote cell attachment (Bruder et al., 1997). Recently, published work looking at the behaviour of MSC cultured on defined chemistries has reported that the cells prefer a NH_2 coating which increased the viability and proliferation rate of MSC, suggesting that this chemistry over the COOH promotes differentiation to the osteogenic lineage. On the COOH defined substrate the cells adhered with high affinity, however the amount of spreading was limited. The contact angles published in this work are however lower than expected for the chemistries used

and suggests that interpretation of the surface chemistry was questionable (Curran et al., 2005). So it is possible that although the acrylic acid coating improves attachment it may be hindering the differentiation of the cells with the surface chemistry used having an inhibitory affect. When choosing a potential coating for biomaterials it is important to get a balance between the enhancement of cell attachment and the affect of the chemistry on the differentiation potential of the cell.

Cell morphology of cells attached to the plasma polymerised coated PDMS substrates were observed to be more spread and fusiform compared to the uncoated substrates. Even though no difference was seen in the numbers of cell attaching, cell morphology on the 1:10 was more comparable to the TCP with the cells on the 1:30 and 1:50 taking more time to spread. However, a spreading assay would be needed to confirm this difference. The observation could be due to the difference in substrate stiffness or could reflect an inadequate coating. However, no significant difference was seen in the growth of the cells over a 10-day period supporting the possibility that the cells take longer to spread on the softer substrates as previously described for fibroblasts and myocytes (Pelham and Wang, 1997) (Engler et al., 2006). In these studies it was shown that cells on softer substrates are more spherical, F-actin more defused with localisation mainly beneath the cell membrane

A major advantage of having substrates that have easily controllable mechanical properties is that a cell population can be studied as a whole. Researchers using micro-fabricated substrates typically use an isolated island for cell adhesion and therefore does not represent a physiological system as a whole. Often viability of the cells and apoptosis levels in these systems are ignored. In the model presented here, no difference in viability was observed after 7 days. Again, this data confirms that although the XPS data demonstrated that the acrylic acid was not necessarily surface coating the 1:50 substrate there is definitely a treatment of some sort that is allowing cells to attach, spread and grow without changing the viability levels of the given population. If the plasma coating had been unsuccessful then low viability levels would have been expected by day 7. The data could however show that once the cells are attached and have presumably laid down their own ECM then the underlying substrate doesn't have such an effect on their viability and by day 7 the cell is able to compensate for any changes in the coating. Viability levels recorded daily over the culture period would

have explored this theory. Furthermore, apoptosis levels were low and although not significant there was a trend for higher levels as the crosslinker ratio increased rendering the substrate softer. This could reflect the fact that cells prefer a stiffer substrate and are able to attach with more affinity or the fact that the coating was insufficient on the softer substrates.

In contrast to findings by Brown et al., (2005) who showed that VSMC proliferated better on softer substrates, we found that MSC proliferated slightly better on the stiffer substrates, although not significantly so. In addition to this, no significant difference was seen between the numbers of MSC in each of the stages of the cell cycle. It has been documented that on polyacrylamide gels, fibroblasts proliferate quicker on stiffer substrates but in this study the range of moduli used was a lot smaller compared to the data presented here (Wang et al., 2000). It has been shown that a difference in cell adhesion can influence the rate of cell division (Danen and Yamada, 2001) highlighting the need for rates of adhesion to be controlled in order for proliferation rates to be assessed accurately.

The high-density culture analysis revealed that ALP levels were significantly higher on TCP compared to the PDMS substrates, suggesting that osteogenic lineage progression is favoured on stiffer substrates. Although this difference was reproducible the variation in ALP levels between the different PDMS substrates were not significant. Furthermore, the reproducibility of this trend was poor. It is possible that the effect seen is due to the integrity of the coating and the loss of the acid functionality as the crosslinker ratio increased (**Figure 4-11**). The result could also be due to a depletion of the coating over time, as highlighted by the XPS data obtained for the stability of the coating (**Figure 4-14**). As cell number was not determined at the end of the culture period it is also possible that there was a difference in cell number although this is unlikely as a confluent monolayer was present at the time of analysis causing any variation to be small. Additionally, there was no change in the rate of proliferation overtime between the PDMS and TCP substrates (**Figure 4-19**). To confirm this result the assay was completed after the cells had been proliferation-arrested to eliminate the effects of proliferation on the assay. Again, ALP levels were significantly higher on the stiffer substrate.

Substrate stiffness had no effect on the CFU-f efficiency as determined by methylene blue staining for total colonies (**Figure 4-24**). This finding supports previous data within this work that showed that cell attachment was consistent onto the coated PDMS substrates regardless of the elastic modulus.

Data gathered for the amount of differentiation to the osteogenic lineage from the CFU-f assay correlates with the high-density culture data, the stiffer the substrate the more ALP positive colonies. This therefore suggests that substrate stiffness does affect the differentiation potential of the MSC. Reproducibility of the plates was found to be questionable and the coating was not always uniform across the whole 55-cm^2 petri dish, with edges not coating stably. This fact could account for the difference seen in osteogenic potential although if this were the case then you would also expect a difference in the total colony number, which was not observed. The data was not significant and is again probably due to the inter-plate variability as the plates were coated in different batches due to limitations with the reactor size. The data is also collated from three independent experiments and therefore differences in the primary cells must be taken into consideration.

At the start of this project there were no published reports of substrate stiffness effecting stem cell differentiation. Previous work had shown that cells do respond to their local microenvironment and that the stiffness of the underlying substrate has effects on not just not cell proliferation but a number of different aspects of cell behaviour including locomotion (Pelham and Wang, 1997) and apoptosis (Wang et al., 2000). More recently, studies have linked substrate stiffness with the differentiation potential of the cell, confirming the data presented here that a stiffer substrate favours the differentiation of MSC to the osteogenic lineage. Furthermore, these studies have demonstrated that the MSC can sense the moduli of the underlying substrate which in turn can influence the differentiation of the cell to the neurogenic, myogenic and osteogenic pathways (Engler et al., 2004b, Engler et al., 2006). Although there is now a link between substrate stiffness and stem cell behaviour it is still a challenge to understand how a cell can perceive the mechanical environment and convert the information into signals to enable changes in cellular function. Even though an exact mechanism remains to be elucidated, earlier studies have linked the cell's cytoskeleton and in particular the non-muscle

myosin ii isoform (Engler et al., 2006) and also the RhoA system (McBeath et al., 2004).

To investigate the role myosin II has on the differentiation of MSC to the osteogenic lineage, non-muscle myosin II was inhibited by blebbistatin. A similar effect was seen as reported by (Engler et al., 2006) in that the addition of blebbistatin post-plating did not significantly alter the morphology of the cells, and suggests that the inhibitor should be added during plating if cell shape is to be altered. Here we report that the addition of blebbistatin to MSC cultured for 7 days in osteogenic medium stimulated the expression of ALP significantly but also removed the difference in the ALP seen on the different substrates. (Engler et al., 2006) found that inhibition of NMM ii blocked all elasticity directed lineage specificity, whilst (McBeath et al., 2004) found that blebbistatin decreased alkaline expression and linked the effect to a change in cell shape.

RhoA is a member of the Ras homology family of small GTPases, with functions predominately linked to cytoskeletal regulation and therefore a number of subsequent cellular functions including stress fibre formation and cytoskeletal tension (Meyers et al., 2005). It has been shown that the use of an adenoviral construct causing RhoA to be constitutively active induces osteogenic differentiation and furthermore a dominant negative RhoA adenoviral constructs leads to adipogenic differentiation. Y27632 is an inhibitor of Rho-kinase (ROCK), a downstream effector protein of RhoA and has previously been used to impair the actin cytoskeleton by specifically inhibiting myosin-generated cytoskeletal tension. Interestingly, we found that the addition of Y27632 to our cultures increased the rate of osteogenic differentiation. This data is conflicting to previously published data by (McBeath et al., 2004) who demonstrated that ROCK inhibition increased the level of osteogenesis linking MSC cell shape to the differentiation potential, with a more spread morphology leading to the induction of osteogenesis and a rounded phenotype inducing adipogenesis.

Investigation of a possible role for the p38 pathway on the effects of substrate stiffness on MSC differentiation was chosen due to its previous connection with the osteogenic differentiation pathway (Jaiswal et al., 2000). We found that inhibiting the p38 pathway decreased ALP activity of the MSC regardless of the stiffness of the underlying substrate confirming that p38 is involved in the differentiation of MSC to the osteogenic

pathway. (Simmons et al., 2003b) found that blocking p38 MAPK activity with SB203580 caused an increase in the ALP activity unless the cells were placed under 3% equibiaxial stain where under these conditions a decrease was seen. Although conflicting this study, with an increase in ALP seen, the addition of the inhibitor removed the difference in ALP expression seen between the PDMS and TCP samples and suggests a possible link between the p38 pathway and MSC being able to sense the underlying substrate with inhibition of the pathway removing the cells ability to distinguish the compliance of the underlying substrate.

4.5 Conclusions

- PDMS offers a convenient artificial flexible substrate. Changing the crosslinking to base ratio allows easy control over the Young's modulus.
- Uncoated PDMS does not support MSC culture typically observed *in vitro*.
- Conventional protein coatings are insufficient to establish attachment and spreading typically associated with MSC in culture.
- The addition of an acrylic acid layer employing plasma polymerisation enhances cell attachment and growth.
- The addition of an acrylic acid layer renders the PDMS hydrophilic.
- The addition of an acrylic acid layer does not alter the mechanical properties of the polymer.
- Substrate stiffness does not affect the rate of proliferation, cell viability or apoptosis levels over a 10-day period.
- The CFU-f efficiency is not altered by substrate stiffness.
- MSC differentiation to the osteogenic lineage is enhanced on stiffer substrates.
- The effect of the substrate stiffness on MSC differentiation is not due to a difference in proliferation.
- Disruption of myosin caused an increase in the expression of ALP regardless of the stiffness of the underlying substrate.
- Inhibition of the Rho-kinase led to an increase in the expression of ALP.
- Inhibition of the p38 pathway led to a reduction in ALP expression.

CHAPTER FIVE: Summary and Final Discussion

In vivo cells are typically attached to a solid substrate, with the suggestion that each cell type has a specific elastic microenvironment that acts with other factors to maintain the phenotype of the cell (Discher et al., 2005). The elasticity of the substrate can range in stiffness from very soft i.e. brain tissue to very hard for in example bone (Liu., 2005). However despite this knowledge, the majority of cell culture *in vitro* is preformed on hard, rigid polystyrene regardless of the cell type, with little consideration given to the effect this environment is having on the cell. Substrate stiffness has now been shown to affect a number of cellular processes including apoptosis (Wang et al., 2000), locomotion (Pelham and Wang, 1997) and more recently the differentiation of stem cells (Engler et al., 2006). These findings highlight the need for further investigation, not just in the understanding of the differentiation process in stem cells, but also for tissue development and repair strategies. Consideration must also be given before stem cells are used in a therapeutic setting, either where they are injected locally, added to a scaffold or introduced via systemic transplantation. In tissue engineering application, where large numbers of cells are needed, culture conditions for the expansion of the cells must also be considered to ensure that the cell's phenotype is maintained with a compromise between convenience and reliability reached.

The aim of the project was to investigate the hypothesis that substrate stiffness will affect MSC proliferation and differentiation. It is only within the past three years that investigators have investigated the effects of substrate stiffness on the differentiation potential of MSC (Engler et al., 2006) (Engler et al., 2004b). In both of these studies polyacrylamide was employed to produce an elastic substratum. As the most characterised flexible substratum system, polyacrylamide offers a number of advantages over other biomaterials as previously described (Wong et al., 2004). However, the range of attainable moduli (E_{max} = ~40 kPa) is a lot smaller than PDMS (E_{max} = ~1.2 MPa). For the study of myogenic differentiation where E_{muscle} = 8-17 kPa, polyacrylamide offers a suitable range but to investigate osteogenic differentiation a larger, more physiological range was needed and is why development of a new system was sought.

To test the hypothesis two systems were developed; a natural fibrin substrate whereby altering the concentration of fibrinogen changes the stiffness of the resultant gel and an artificial silicone substrate where the Young's modulus can be controlled by altering the degree of crosslinking within the polymer. Mechanical testing and surface science

techniques allowed characterisation of the systems before biological assessment was performed to ensure that the substrates supported cell attachment and growth.

The fibrin gels were able to support monolayer culture without any further modifications. Although not significant, initial cell attachment was seen to be higher on the fibrin gels compared to the standard TCP with the rate of proliferation higher on the softer gels for up to 7-days in culture. Viability and apoptosis levels were not significantly different over the 11-day culture period. The morphology of the cells demonstrated a trend that as the stiffness of the fibrin increased a more elongated, fibroblastic morphology was seen. This trend was not seen however on the TCP where the typical "cobblestone" morphology of the MSC in culture was observed.

After a selection period and an initial culture on the fibrin gels the substrate type was seen to alter CFU-f efficiency with a trend for an increase in the number of colonies after a pre-culture on the softer substrates. Furthermore, the percentage of cells staining positively for ALP, a common osteoblastic marker, was higher on the softer substrates and suggests that pre-culture on softer substrates supports the maintenance of a more "stem cell" like phenotype. The effect was the same for both primary and early passage cells. This finding was linked to the size of the cell as analysis of the forward scatter showed that MSC cultured on the softer gels maintained a smaller cell size. In addition, it was shown that osteogenic differentiation was favoured on stiffer substrates and furthermore it was demonstrated that adipogenesis was increased on the softer substrates supporting the hypothesis that the mechanical properties of the underlying substrate can alter the differentiation potential of the MSC. One limitation of the fibrin system was that the effect of the protein concentration must be considered and highlighted the advantage of an artificial system whereby a substrate with clearly defined chemistries can be produced.

The data presented here shows that fibrin is a good choice of biomaterial for the study of a more physiological environment on stem cells behaviour. The CFU-f data suggests that pre-culture on a softer substrate leads to the maintenance of a more 'stem cell' like phenotype. Overall the results suggest that culturing MSC on fibrin gels affects their initial growth without changing the high viability levels typically seen on TCP. The

softer substrate enables the cells to orient themselves better whilst maintaining a smaller cell size.

The results question the ideal expansion conditions for MSC *in vitro* and suggest that the standard TCP is not necessarily the ideal substrate if a multipotent phenotype is to be maintained. The results also have important implications in tissue engineering applications especially in scaffold design. A scaffold produced or coated with a soft biomaterial could lead to the retention of a more 'stem cell' phenotype of the cells incorporated within the structure. These data also highlight the point that numerous factors need to be taken into consideration to assess the suitability of different biomaterials and that attachment and growth alone should not just be used; cell orientation, shape and size should be considered too.

PDMS can be produced with a much large range of mechanical properties compared to the fibrin. However, uncoated PDMS does not support MSC attachment and growth that is typically seen *in vitro.* The pre-absorption of a protein layer did not enhance cellular attachment or spreading and therefore plasma polymerisation was employed to surface coat the PDMS with a nano-thin layer of acrylic acid. Mechanical testing confirmed that the addition of this layer did not alter the mechanical properties of the substrate. Although XPS analysis was unable to show that a complete coating was added to all of the substrates, contact angle analysis demonstrated that the substrates were rendered more hydrophilic after the addition of the acrylic acid layer and the PDMS subsequently supported MSC attachment and growth. Once coated the growth of the cells were comparable to growth on TCP over a 10-day culture period with no differences in cell viability or apoptosis levels.

Substrate stiffness did not directly affect CFU-f efficiency but a trend for differentiation to the osteoblastic lineage was greater on the stiffer substrates. The increase in osteogenic differentiation on-high modulus matrices was not due to a reduction in cell proliferation. Data regarding a possible mechanism of action was inconclusive but demonstrated the involvement of the non-muscle myosin II and the RhoA pathway in the mechanotransduction pathways.

Overall, the study established that PDMS coated with acrylic acid is a good choice for a biomaterial in the study of the elastic properties of the underlying substrate on cell behaviour. A large range of Young's modulus can easily be produced and a nano-thin coating of acrylic acid does not significantly affect cell proliferation, viability or apoptosis levels. Furthermore, using the model we were able to demonstrate that differentiation to the osteogenic lineage is favoured on a stiffer substrate again confirming published data (Engler et al., 2006). An exact mechanism of action remains to be elucidated.

The work presented here proves that the mechanical properties of the underlying substratum can affect stem cell behaviour. Potentially a small variation can cause a change in cellular function including the end phenotype of a stem cell. These data have important implications for tissue engineering applications and could potentially lead to the development of biomaterials with defined mechanical properties that could positively influence cells cultured upon it. The elimination of expensive growth factors and other artificial soluble factors to either direct or preserve the differentiation potential of the MSC would be a significant advance within the field.

Further Directions

This data presented here raises a number of questions that would require further investigation:

- In the presented work, the surface chemistry of the PDMS substrates was intended to be controlled. However, the XPS data for the 1:50 PDMS substrate revealed that more silicon was present than compared to the other samples. Although no difference in the contact angle measurements was observed, it is possible that this difference in substrate chemistry could affect the growth and differentiation of the cells. Therefore this raises the question, "Is the differentiation potential of a cell influenced directly by the underlying chemistry'? and if so 'can the cell actually sense the different chemistry or is it through a difference in the surface energy and subsequent degree of protein adsorption?" To investigate these points further, a number of surface chemistries could be produced employing plasma polymerisation and characterised thoroughly using a number of surface science techniques. The effect on protein adsorption and cellular function including differentiation could then be investigated.

- Furthermore, "is the topography and substrate structure affecting the differentiation potential?" as previously described for other aspects of cellular behaviour. SEM and AFM analysis of the surfaces would enable the nano-structure including the topography to be considered.

- The differentiation potential of stem cells has previously been linked to cell shape. In the work presented here, a change in morphology was observed with the variations in substrate stiffness, it is feasible that the change in substrate stiffness was having an effect on MSC shape, which could have correlated to the change in differentiation potential. To explore this notion further it would be interesting to monitor closely the change in cell morphology, especially the organisation and number of FAK complexes which link the underlying substrate to the cell's cytoskeleton.

- Carrying on from the last point, completing spreading assay and morphology profiles to analysis subtle changes in the cell morphology could possibly link these data to previous studies demonstrating that MSC can be further sub-divided into a number of sub-populations which subsequently effects the differentiation potential of the cell population.

- For tissue engineering application to be come reality large number of cells are needed. Within this work it has been demonstrated that MSC expand quicker on softer, more physiological substrates whilst maintaining their osteogenic differentiation potential. This raises the question, "would a flexible, more physiological substrate affect long-term culture of MSC?" MSC are known to have a finite lifespan that questions their "stem cell" classification. Would this lifespan be improved by long-term culture on softer substrates? It would be interesting to complete a long-term growth curve on substrates with different stiffnesses to establish the number of attainable population doubling, to see if a softer more physiological environment had any effect on the proliferation and differentiation potential of MSC.

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Appendix one:

Flow rate calculation for plasma polymerisation

$PV = nRT$

$n = PV/RT$

$dn/dt = d/dT = Pv/ Rt$

Flow rate, $F = dn/dt = dp/dt\ (V/RT)$ mol/sec

$F = dp/dt\ (V/T)$ 16172 cm^3 STP/min

$F = d\ (P_1-P_2) \times 16172/ T \times 15.121/30$

$F = d(P_1-P_2)$ 27.82

Where:
n = moles of gas
P = pressure (N/m^2)
V = volume of reactor (15.121 L)
t = time
R = gas constant
T = temperature (K)

Appendix two:

	Statistically significant difference
Day 2	• 3 mg/ml –30 mg/ml • 3 mg/ml-TCP
Day 3	• 3 mg/ml –30 mg/ml • 3 mg/ml-TCP • 10 mg/ml-30 mg/ml
Day 4	• 3 mg/ml –30 mg/ml • 3 mg/ml-TCP
Day 7	• 3 mg/ml –10 mg/ml • 3 mg/ml –30 mg/ml • 3 mg/ml-TCP • 10 mg/ml-30 mg/ml • 10 mg/ml-TCP
Day 9	Not significant
Day 11	Not significant

Table 7-1: A table to shown the statistical analysis for the growth curve for MSC cultured on fibrin substrates (Figure 3-3), determined by ANOVA (p=<0.05).

	Statistically significant difference
Day 2	• 30 mg/ml-3 mg/ml • 30 mg/ml- 10 mg/ml • 30 mg/ml-TCP
Day 3	Not significant
Day 4	Not significant
Day 7	Not significant
Day 9	Not significant
Day 11	• 3 mg/ml-TCP • 3 mg/ml-10 mg/ml • 3 mg/ml- 30 mg/ml • 10 mg/ml-TCP

Table 7-2: A table to shown the statistical analysis for the viability of MSC cultured on fibrin substrates (Figure 3-3), determined by ANOVA (p=<0.05).