Investigation into the Incorporation of RGD into Polymers as a Non-Integrin Selective Strategy for Tissue Engineering

Lynne Perlin

A Dissertation submitted for the Degree of Doctor of Philosophy.

Department of Chemistry and
Department of Materials Engineering

April 2007

// Acknowledgements

I would like to thank all the people who have made this research possible:

- My supervisors Dr Steve Rimmer and Professor Sheila MacNeil.
- My colleagues in both research groups for their invaluable help
- The technicians for setting up, maintaining and repairing the equipment.
- The donors of the human cells used in this project.
- The EPSRC for the financial support
- My partner Will Bayley for keeping me going, making me happy and looking after me.
- My parents and my sister for their years of patience whilst trying to be interested, always being there to talk to and providing unfailing support in every way.

Abstract

This thesis describes the development of the 4-bromobenzylsulphonyl (4-Bbs) group as an enzymatically cleavable protecting group for the side chain of arginine. The protected arginine was utilised to synthesise RGD peptides both with and without a spacer arm and these peptides were incorporated into hydrogels. Hydrogels were made from 1,2-propandiol-3-methacrylate (glycerol methacrylate, GMMA) or butyl methacrylate (BMA) and 1,2-ethandiol dimethacrylate (ethylene glycol dimethacrylate, EGDMA) and photopolymerized as 60μm coatings. Removal of the 4-Bbs protecting group was achieved by incubation with the enzyme Glutathione-S-Transferase.

Culture of human dermal fibroblasts on the materials showed significant improvements in cell adhesion and viability in serum free media on glycerol methacrylate hydrogels with nominal RGD concentrations of 1μmol/g or greater. A spacer arm between the peptide and the bulk was not necessary to promote cell attachment. No significant improvement in cell adhesion and viability to butyl methacrylate hydrogels was observed at any of the peptide concentrations tested. The effects of peptide concentration, GST pre-treatment of materials, cell passage number and culture with soluble RGD were investigated by examining cell morphology, adhesion, viability and F-actin organisation.

Contents

1 Aims and Hypotheses 1

2 Definitions and Abbreviations 6

3 Introduction 9

3.1 Biomaterials for Tissue Engineering 9

3.1.1 Requirements for Biomaterials 9

3.1.2 Approaches to Tissue Engineering 10

3.1.3 Types of Materials used for RGD Modification 12

3.1.4 Protein Adsorption on Biomaterials 15

3.2 Materials Design and Synthesis 20

3.2.1 Physical Methods 21

3.2.2 Chemical Methods 1: Modification 25

3.2.3 Chemical Methods 2: Incorporation 38

3.2.4 Assessment of RGD Concentration 40

3.3 Assessment Methods and Their Effect on Outcomes 44

3.3.1 Cell Behaviour 45

3.3.2 Experimental Design 50

3.3.3 Analysis Methods 57

3.4 Effects of RGD Design on Cell Behaviour 62

3.4.1 Material Design Effects 62

3.4.2 RGD Sequence 77

3.4.3 RGD Concentration 83

3.4.4 Other Peptide Sequences 90

3.5 Conclusions 92

4 Experimental Design 96

4.1 Objectives 96

4.2 Design Criteria 97

4.3 Experimental Design 98

5 Results & Discussion 101

5.1 Polymerisation Techniques 101

5.1.1 AIBN Initiation 101

5.1.2 UV Initiation 104

5.1.3 Summary of Advantages & Disadvantages 111

5.2 Peptide Incorporation Techniques 112
5.2.1 Strategy 1: TFA Deprotection of Side Chains 112
5.2.2 Strategy 2: Enzyme Deprotection 115
5.3 Amino Acid Synthesis and Deprotection 117
5.3.1 Synthesis of Z-Arg(4-Bbs) 118
5.3.2 Determination of Deprotection by Sulphite Assay 119
5.3.3 Determination of Deprotection by HPLC 123
5.3.4 Synthesis of Fmoc-Arg(4-Bbs) 127
5.3.5 Deprotection of Fmoc-Arg(4-Bbs) 128
5.4 Peptide Synthesis 130
5.5 Polymer Library Synthesis and Deprotection 135
5.5.1 Strategy 1: TFA Deprotection of Peptides 135
5.5.2 Strategy 2: Enzyme Deprotection 136
5.6 Analysis of Cell Behaviour on Polymers 140
5.6.1 General Information 140
5.6.2 Serum-Containing Media 141
5.6.3 Effect of GST on Cell Culture 155
5.6.4 Reaction of Peptide with Serum 158
5.6.5 Cell Culture in Serum Free Media 160
5.6.6 Peptide Concentration Analysis 167
5.6.7 Competition with Soluble RGD 172
5.6.8 Peptide Deprotection by Non-contacting Cells 176
5.6.9 Passage Number 180
5.6.10 Histology 182
6 Conclusions & Future Work 194
7 Materials and Methods 201
7.1 Monomer Synthesis 201
7.1.1 Synthesis of GMAc 201
7.2 Polymer Synthesis 201
7.2.1 Materials 201
7.2.2 Linear Polymer Synthesis 202
7.2.3 Hydrogel Synthesis by Thermal Initiation 202
7.2.4 Hydrogel Synthesis by UV Initation 203
7.3 Polymer Reactions 204

7.3.1 Reaction of Linear Polymers with TFA 204
7.3.2 Reaction of Cross-linked Polymers with TFA 204
7.4 Amino Acid Synthesis 205
7.4.1 Materials 205
7.4.2 Synthesis of Z-Arg(4-Bbs) 205
7.4.3 Synthesis of Boc-Arg(4-Bbs) 207
7.4.4 Synthesis of H-Arg(4-Bbs) 209
7.4.5 Synthesis of Fmoc-Arg(4-Bbs) 211
7.4.6 Synthesis of Fmoc-ADDA 212
7.5 Synthesis of Peptide Sequences 213
7.5.1 Materials 213
7.5.2 Resin Loading 213
7.5.3 Deprotection and Monitoring 214
7.5.4 Protected Peptides 214
7.5.5 Non-Protected and Partially-Protected Peptides 215
7.5.6 Analysis and Purification of Peptide Sequences 216
7.6 Investigation of GST deprotection 218
7.6.1 Materials 218
7.6.2 Determination of GST Deprotection by Sulphite Assay 218
7.6.3 Determination of GST Deprotection by HPLC 219
7.7 Peptide-Containing Hydrogel Synthesis 220
7.7.1 Synthesis of polymers 220
7.7.2 Enzyme Deprotection of Peptides 220
7.8 Cell Culture 220
7.8.1 Cell Maintenance 221
7.8.2 Cell Culture on Polymers 221
7.8.3 Competition with Soluble RGD 221
7.8.4 Incubation with Non-Contacting Cells 222
7.8.5 MTT Assay 222
7.8.6 Histology 222
8 References 224

1 Aims and Hypotheses

Novel biomaterials that come into contact with anchorage dependent cells can be divided into two general groups, those that are intended to support the attachment of the cells and those that are designed to resist the attachment. Of the materials where it is desirable to support the attachment of cells there are two main types, those that possess functionalities that bind directly to cells and those that rely upon protein adsorption and subsequent binding of the proteins to cells.

The adhesion of proteins to materials allows the direction of cell attachment and growth by the non-specific adsorption of a wide range of proteins from serum in the media and / or cell derived products. The non-specific adsorption of proteins has the advantage that cells perceive an environment where they have access to a large range of growth factors and signalling mechanisms to promote normal cell behaviour. The disadvantages are that the processes are improperly understood and therefore the results may be variable. Also non-specific adsorption removes the ability to control and direct cell growth and functions into areas dictated by the needs of tissue engineering. In addition the use of serum from animal sources is controversial as there are possibilities of viral transfection.

Functionalised biomaterials have the potential to direct cell growth and behaviour. These attributes may make it possible to encourage better integration of implantable biomaterials and improve the culture of cells *in vitro* without the use of serum. Background materials that resist protein adsorption allow materials with well-defined and controllable properties. However the resistance of a substrate to protein adsorption may result in cells being unable to access necessary growth factors or signalling mechanisms.

Peptides containing the sequence RGD are known to bind to integrins and to promote the adhesion of cells to biomaterials in which they are incorporated. Different flanking sequences affect the degree of affinity of the peptide for different integrins leading to the ability to confer selectivity onto the material, for example some cyclic RGD containing peptides can only bind to integrins found in osteoblast cells. However most cell types do not possess an integrin by which selective adhesion can be mediated such that in the majority of cases wherein

RGD is utilised it is in a non-selective manner. This gives rise to a number of questions on the usefulness of RGD peptides:

1. If the adhesion of cells to the substrate is non-integrin selective is there value to be obtained in combining the two strategies of supporting cell adhesion, namely in promoting cell adhesion by RGD and supporting cell functions by protein adsorption to the substrate?
2. Does the ability of RGD to promote cell adhesion hold in the case where protein adsorption is present, i.e. are the processes competitive or cumulative?
3. Does the use of RGD peptides confer any value on the substrate that is not obtainable by protein adsorption?

In summary "Is the incorporation of RGD peptides into polymeric biomaterials a useful tool for promoting non-selective cell adhesion for tissue engineering purposes?"

Hypotheses

The processes of cell adhesion to substrates via protein adsorption and RGD modification will be competitive. On materials that are not able to effectively mediate cell attachment by protein adsorption the inclusion of RGD will result in a large increase in cell attachment. On substrates that are better able to support cell attachment by protein adsorption the inclusion of RGD will cause an improvement in cell attachment but the magnitude of the improvement will be less than on the poorly adhesive materials. On substrates that are able to support high levels of cell attachment by protein adsorption the inclusion of RGD will not improve cell attachment. A hypothetical model of how the incorporation of an RGD peptide would improve the ability of substrates to adhere cells is shown in Figure 1-1.

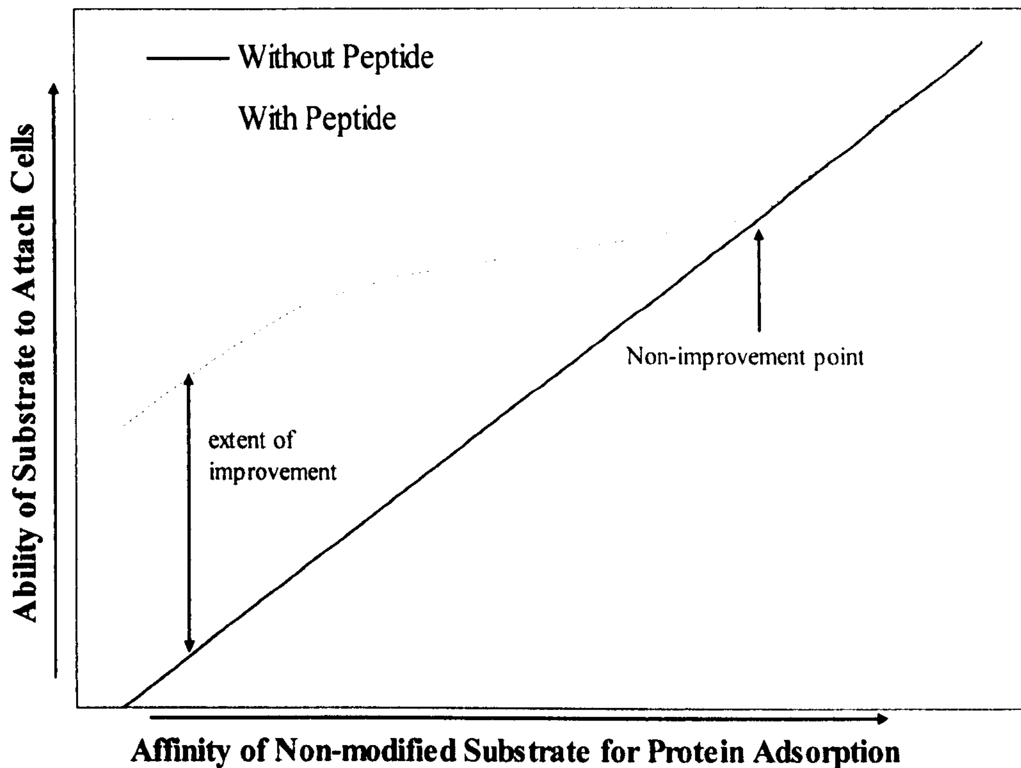


Figure 1-1 Hypothetical model of how the incorporation of an RGD peptide would improve the ability of substrates to adhere cells.

4. If the nature of the peptide and the concentration and type of integrins are kept constant the extent of improvement and the non-improvement point will depend on the concentration of the peptide. With a higher peptide concentration the extent of improvement will increase and the non-improvement point will be advanced such that peptide inclusion will provide improvement at higher intrinsic level of cell attachment as illustrated in Figure 1-2.

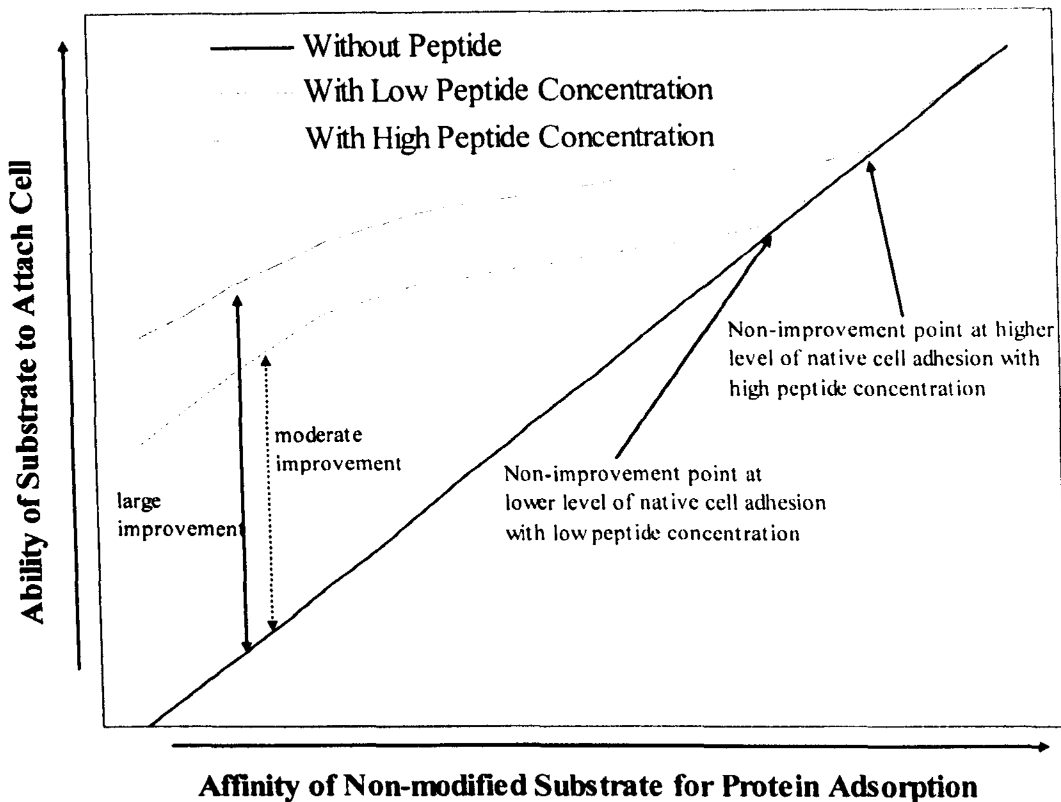


Figure 1-2 Hypothetical model of how the incorporation of an RGD peptide would improve the ability of substrates to adhere cells at low and high concentrations of peptide.

5. The extent of improvement and the non-improvement point will be advanced by increased integrin binding. Increased integrin binding can be achieved through use of more affine ligands, use of cells with greater integrin density or use of cells wherein the integrin type has a greater affinity for the peptide utilised.
6. The effectiveness of the inclusion of a RGD peptide will decrease with time as a surface becomes remodelled and supports greater protein adsorption.

If these hypotheses are proved correct there are several implications for the use of RGD in substrates designed for tissue engineering purposes:

1. RGD incorporation will only be an effective strategy in materials that do not adsorb proteins to a significant degree.
2. Higher concentrations of peptide will be more effective at promoting cell adhesion in the same substrate. The minimum concentration of peptide required to elicit a significant effect will increase as the ability of a substrate to adsorb proteins increases.
3. The effectiveness of the inclusion of an RGD peptide will decrease with increasing passage number for primary cells. The integrin expression profile of a cell type will determine how effective RGD inclusion will be. The use of RGD peptides that are more affine to a particular cell type will promote a larger improvement and allow the use of smaller peptide concentrations to achieve a significant effect.
4. The inclusion of RGD peptides will be effective at promoting short term adhesion but will not be an effective strategy for long-term cell survival and normal functioning without the ability for the background substrate to adsorb proteins. The inclusion of RGD peptides may be an effective strategy in combination with other functionalities such as growth factors that promote the normal behaviour of cells in different aspects other than the short term integrin-mediated cell attachment.

2 Definitions and Abbreviations

These definitions are used in this text for purposes of clarity for a current and future interdisciplinary readership.

- Modification: any procedure in which a peptide-containing species is chemically attached to a pre-existed material in situ
- Incorporation: any procedure in which a peptide-containing species is chemically attached to a substrate as that substrate is being formed
- Coating: any procedure wherein the peptide-containing species is not chemically attached to a material in situ
- Blend: a coating procedure where an RGD-containing species is incorporated into a material as it being formed by non-covalent means.
- Coupling reagent: any species that mediates the attachment of the peptide without leaving a residue in the final product.
- Cross-linker: any species that mediates the attachment of the peptide and leaves a residue in the final product.
- Specificity / specific: effects caused by the binding of RGD to integrins rather than through other mechanisms e.g. protein adsorption.
- Selectivity / selective: effects caused by the binding of an RGD sequence to a particular integrin type
- Scrambled RGD peptide: a peptide sequence of any length that contains arginine, glycine and aspartic acid next to each other but not in the correct RGD sequence to form an active peptide
- Substituted RGD peptide: a peptide sequence of any length that contains a tripeptide sequence similar to RGD that has one of the amino acids substituted with another that is similar in structure to form a non-active sequence

Abbreviation	Full name
AA	Acrylic acid
AIBN	Azobisisobutironitrile
BMA	Butyl methacrylate
BSA	Bovine serum albumin
CIPAAM	2-carboxyisopropylacrylamide
DCC	Dicylcohexylcarbodiimide
DIPEA	Diisopropylethylamine
DMAEM	Dimethyl aminoethyl methacrylate
DMAP	4-dimethylaminopyridine
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DVB	Divinyl benzene
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGDMA	1,2-ethandiol dimethacrylate, Ethylene glycol dimethacrylate
ELISA	Enzyme-linked immunosorbent assay
GMMA	1,2-propandiol-3-methacrylate, Glycerol mono methacrylate
GSH	Reduced glutathione
GST	Glutathione-S-Transferase
HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	N-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
LC-SPDP	N-succinimidyl-6-(3-[2-pyridyldithio]-propionamido)hexanoate
LMA	Lauryl methacrylate
MES	2-Morpholinoethanesulfonic acid
MSNT	1-(Mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole
NHS	N-hydroxy succinimide
NHS	N-hydroxy succinimide
NIPAAM	N-isoproylacrylamide

NIPAM or NIPAAM	N-isopropyl acrylamide
NMR	Nuclear magnetic resonance
OWLS	Optical waveguide lightmode spectroscopy
PBS	Phosphate buffered saline
PCS	Peptide-containing species
PEG	Poly ethylene glycol
PET	Polyethylene terephthalate
PLGA	Poly(L-glycolic acid)
PLLA	Poly(L-lactic acid)
PTFE	Polytetrafluoroethylene
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
RGD	Arginine-Glycine-Aspartic Acid or longer peptide sequence containing this active moiety
SAM	Self-assembling monolayer(s)
SANPAH	N-Succinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate
SMBP	N-succinimidyl-4p-maleimidophenyl-butirate
SMCC	N-succinimidyl trans-4-(maleimidylmethyl)cylcohexane-1-carboxylate
SMP	N-succinimidyl-3-maleimidopropionate
SPDP	*N*-succinimidyl-3-(2-pyridyldithio)-propionate
TCP	Tissue culture plastic
TsU	N,N,N',N'-tetramethyl(succinimido)uranium tetrafluoroborate
UV	Ultra-violet

3 Introduction

3.1 Biomaterials for Tissue Engineering

The use of cell culture for standard applications is well established but most techniques are based on the "cell-derived matrix model" where the substrate only provides sufficient attachment for the cells to produce their own extra-cellular matrix. The culture of cells on these substrates generally results in rapid growth but the replication ceases upon contact such that the cells form, at best, monolayer coverage of the substrate rather than the three-dimensional matrixes of natural tissue[1,2]. In addition many of the natural functions of cells are lost or altered once they are removed from their native environment such as protein secretion, respiratory capacity and other functions such as the internal potassium levels. There is also a growing awareness of the need to reduce the amounts of animal derived products, such as fetal calf serum, in tissue engineering to minimise the risks of infection. In consequence there is a considerable interest in producing materials that can modulate and direct the functions of cells for use in tissue engineering.

3.1.1 Requirements for Biomaterials

The requirements of a material intended for use in tissue engineering vary dramatically depending on the specific purpose for which they are intended. Areas that are currently being investigated as targets for tissue engineering include skin, cartilage, bone, vascular, nerve and liver regeneration[3-5].

The physical properties that may be of importance include strength (yield, tensile, compressive, shear, flexural, fatigue, impact and adhesion), resistance (creep, friction, wear, fatigue, abrasion), elastic modulus, shear modulus, stiffness and hardness. Other physical properties may become important in specific applications, for example colour, opacity and refractive index become important in the production of artificial corneas[6]. The biological properties that may be of importance include lack of adverse effects (non-toxic, non-irritant, non-allergenic, non-carcinogenic, non-thrombogenic)[7], the promotion of beneficial effects (cell adhesion, migration, growth and differentiated function)[8] and the interaction of the material with its surroundings (protein adsorption,

initiation of immune response and biodegradation). Chemical properties include biostability (resistance to corrosion, hydrolysis and oxidation) and biodegradation and the chemical composition both in bulk and at the surface affects the physical and biological properties[9]. In addition the processing and fabrication of the material must be considered in terms of reproducibility, quality and sterilizability[10].

The tissue engineering of skin poses a simpler challenge than that of most other tissues, as the structure is a sheet rather than a 3D structure. Serious damage to the skin, which affects the dermal and sub dermal layers of the skin as well as the upper epidermal layer, can cause many problems such as severe dehydration and infection. The body cannot heal these multi-layer wounds properly and grafting is required to replace the skin. Auto grafting is problematic as the donor site becomes a new wound and finding a donor site can be difficult when the area of damage is extensive. Disease transmission and adverse immune responses are problems with cadaver skin grafting and xenografting techniques. Consequently a large number of researchers are working towards therapies that regenerate or help the body to regenerate the dermis, epidermis or full-thickness of skin.

3.1.2 Approaches to Tissue Engineering

The development of novel materials for cell culture and tissue engineering has been a fertile area of research with many different approaches being taken to increase the effectiveness of the substrate. The only common factor that links the various researches into materials designed for tissue engineering is the stipulation that the products that come into contact with cells must be non cytotoxic and provide a solid support for the attachment of cells. The approaches that are used to encourage cell adhesion are varied and make use of a wide range of materials that impart varying properties to the substrate.

For the purposes of tissue engineering with anchorage dependent cells the material used as the substrate must be a solid for the duration of the time that cells are in contact with it. Some polymers can be utilised in liquid form, to allow injection of material or encapsulation of cells and later solidified[11,12]. Other polymers may originally be solid but degrade under physiological conditions thus leaving a space for replacement tissue[13].

Amphiphilic polymers mimic the extracellular matrix's material properties and high water content. The cell adhesion is controlled by adsorbed proteins, which are in turn regulated by the surface properties of the polymer network[14-17]. Studies have shown that both the degree of adsorption and the conformation of the protein are directed by the surface's properties[15,16]. Increased hydrophilicity of the surface (determined by contact angle)[14] and increased bulk hydration (water content)[17] decrease the extent of protein adsorption and hence the utility of the material as a cell culture substrate. However there is evidence that a minimum hydration is necessary to allow cell adhesion and that polar groups contained in networks with low hydration (caused by extensive cross-linking) increase the amount of protein adsorption[17]. The amount of an adsorbed protein does not determine the extent of the biological activity. A protein that has been adsorbed in low concentrations may present more favourable conformations for an increased percentage of protein bound to integrins or an increased binding strength[16].

Glass, metals and some polymers are static systems where the surfaces are fixed into place. Polymers are often highly solvated under culture conditions and can possess very mobile chains that can dynamically re-arrange under changing conditions (e.g. on hydration to cell culture conditions). The dynamic nature of the surface may affect the adsorption of proteins and the ability of cells to adhere to a substrate.

Another strategy utilised to increase the effectiveness of materials used for tissue engineering is to include peptide sequences with active moieties to promote some aspect of cell behaviour. The tripeptide sequence Arginine-Glycine-Aspartic acid (RGD) is often used to promote cell attachment and spreading as it binds to transmembrane proteins called integrins that are found in most mammalian cells. The integrins contain two receptor sites that bind to a peptide adhesion sequence outside the cell membrane and to the protein talin on the internal side of the cell membrane. Talin is in turn linked to actin filaments of the cell via the protein vinculin such that the binding of the adhesion sequence induces a change in the conformation of the cytoskeleton to promote cell adhesion and spreading. The adhesion of cells to substrates affects other cellular characteristics and mechanisms (for example migration and protein expression)

such that the binding of integrins to RGD peptides can affect many different aspects of cell behaviour.

3.1.3 Types of Materials used for RGD Modification

The processing of materials can generate different shapes and forms such as sheets, membranes, spheres[18,19] and three-dimensional structures. Of considerable interest is the production of three-dimensional scaffolds to aid the development of vascularisation[20], cellular in-growth and three-dimensional tissue structure[21]. In addition these 3D scaffolds can be utilized as *in vitro* models of tissue and to study relationships between structure and function[22]. Examples of 3D scaffolds that have been functionalised with RGD include chitosan[23], poly(D,L-lactide-co-glycolide)[24-26], fibrin[27] and collagen[28]. The 3D nature of the scaffold can be developed through foaming, freeze gelation[23] and electro-spinning of linear polymers[26]. Some images of 3 dimensional structures that can be formed from polymers for use in cell culture can be found in Figure 3-1 below.

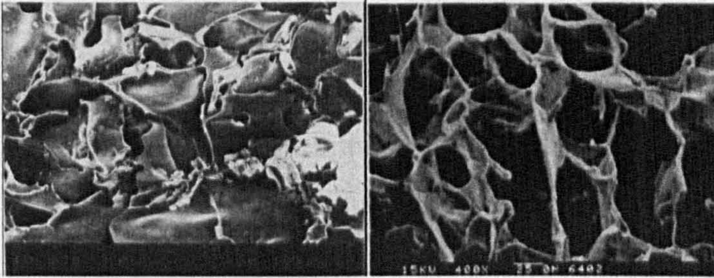


Figure 3-1 Left: Morphology of the chitosan scaffold prepared by the freeze gelation method (SEM, x200) after RGDS immobilization, reproduced from Ho et al[†,23]. Right: Scanning electron micrograph showing the porous structure of PLLA scaffolds (surface view), reproduced from Hsu et al. 2006[‡,24]

Also of interest is the encapsulation of cells as the material is formed by photo-polymerisation[27,29-37], this poses challenges in that the precursors must be non-cytotoxic and the material must be formed under sterile conditions[38].

† Reproduction of Figure 1 from "Ho, M.-H., Wang, D.-A., Hsieh, H.-J., Liu, H.-C., Hsien, T.-Y., Lai, J.-Y. & Hou, L.-T. Preparation and characterization of RGD-immobilized chitosan scaffolds. *Biomaterials* **26**, 3197-3206 (2005)", with permission from Elsevier Ltd.

‡ Reproduction of Figure 2 from "Hsu, S.-H., Chang, S.-H., Yen, H.-J., Whu, S. W., Tsai, C.-L. & Chen, D. C. Evaluation of Biodegradable Polymers Modified by Type II Collagen and Arg-Gly-Asp as Tissue Engineering Scaffolding Materials for Cartilage Regeneration. *Artificial Organs* **30**, 42-55 (2006)", with permission from Blackwell Publishing.

All background materials modified with bioactive functionalities are required to be biocompatible, which effectively means that they must all exhibit a lack of adverse effects at the site of application during the timescale of their use[39]. In addition the substrate can be either bioinert or bioactive. Substances that are desired to be bioinert are required to have as little effect on the biological system as possible and to resist protein deposition and remodelling of the surface. These materials are often derivatised with RGD peptides and are generally intended to investigate the pathways of specific RGD-mediated cell adhesion[40] or in situations where non-specific adhesion is a problem such as re-endothelialisation *in vivo*[41]. Bioactive materials can interact with the biological system either directly through chemical groups on the surface or indirectly (by protein deposition). In some cases the substance that provides the mechanical properties may be completely masked by modifications or coatings.

The types of materials that fit requirements for tissue engineering and are most often used with RGD-peptides fall into two main categories, polymers and inorganic materials.

The polymers utilised in tissue engineering can be derived from natural sources or can be made synthetically. Some examples of naturally derived polymers that have been modified with the RGD-peptide are albumin, alginate, cellulose, chitosan, collagen, dextran, gelatin, glycosaminoglycan, fibrin, halouronic acid, keratin and streptavidin[23,36,42-51],[52,53].

There is a large amount of literature related to RGD-containing synthetic polymers, which may be linear, branched, or cross-linked. Linear and branched polymers are usually mounted onto a suitable substrate, such as tissue culture plastic or glass, before assessment as their physical properties are not generally suitable for handling without support. There are two main methods of synthesizing polymers: step-growth polymerisation and chain growth polymerisation.

The backbone of polymers synthesised by step-growth polymerisation are most commonly formed of ester, amide, or urethane bonds as shown in Figure 3-2.

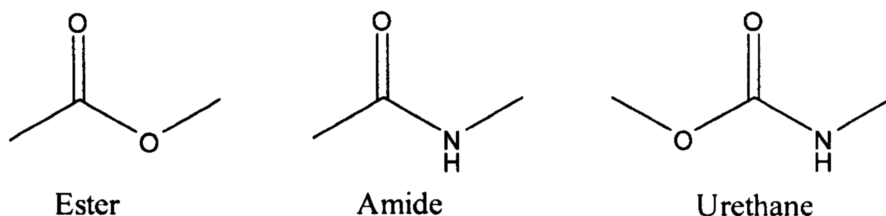


Figure 3-2 Common backbone linkages of step-growth polymers

Examples of esters include polyester[54], poly(lactic acid)[55], poly(lactic-co-glycolic acid)[56] and poly(caprolactone)[57-59]. Examples of amides include poly (L-Lysine)[60]. Examples of urethanes include polyurethane[61-63], polycarbonate urethane[64,65] and polyether urethane[66].

Chain growth polymers are synthesised by the use of an initiator to instigate the formation of the chain. These initiators may be free-radical, cationic or anionic depending on the choice of monomer and desired properties of the resultant polymer. In addition the initiator may be activated by heat, electromagnetic radiation, or chemical reaction. Some monomers that are used to make chain growth polymers that have been utilised as RGD-containing biomaterials include acrylic acid[67] acrylamide[68-72], N-isopropylacrylamide[18,32,33,73-76], methyl methacrylate[77-80], hydroxyethyl methacrylate[81,82], hydroxypropyl methacrylate[83-85], aminoethyl methacrylate[18], vinyl alcohol[86], ethylene glycol vinyl ether[87] and styrene (as in tissue culture polystyrene)[88-93]. The most utilized synthetic polymer appears to be poly(ethylene glycol) (PEG) with at least 30 articles published using this polymer as one of the components[18,30-34,40,41,55,80,81,94-115]. PEG can be obtained with various chain lengths (and hence various molecular weights) and also can be derivatised with various end groups such as acrylates[31,41,98,99], amines[80,105] and carboxylic acids.

Inorganic materials that have been derivatised with RGD-peptides include glass, ceramic, metals and inorganic polymers. Metals that have been either coated with or had the peptide sequence attached to them include titanium[116-119] and gold[118]. Inorganic polymers including silicon[120], silicone[121] and alumina[122]membranes have been utilised for RGD-modification. Glass has been frequently derivatised with RGD peptides[123-127,128-131,132].

3.1.4 Protein Adsorption on Biomaterials

The responses of cells cultured on biomaterials *in vitro* or in contact with biomaterials *in vivo* is controlled or affected by the adsorption of proteins on the surface. In the majority of cases materials being investigated or utilised *in vivo* are exposed to a wide range of proteins from serum in media, cell-derived proteins or body fluids such as blood or plasma. The roles and mechanisms of each of these proteins in controlling cell behaviour singly and in combination are still under investigation by researchers however there are several families of proteins that are known to promote cell adhesion to extracellular matrix or to other cells.

3.1.4.1 Adhesion Proteins

Cells produce surface proteins called CAMs that regulate the adhesion of cells to other cells of the same type (homophilic adhesion), to cells of a different type (heterophilic adhesion) or to the extracellular matrix. There are 5 principal classes of CAMs: cadherins, the immunoglobulin (Ig) superfamily, selectins, mucins, and integrins.

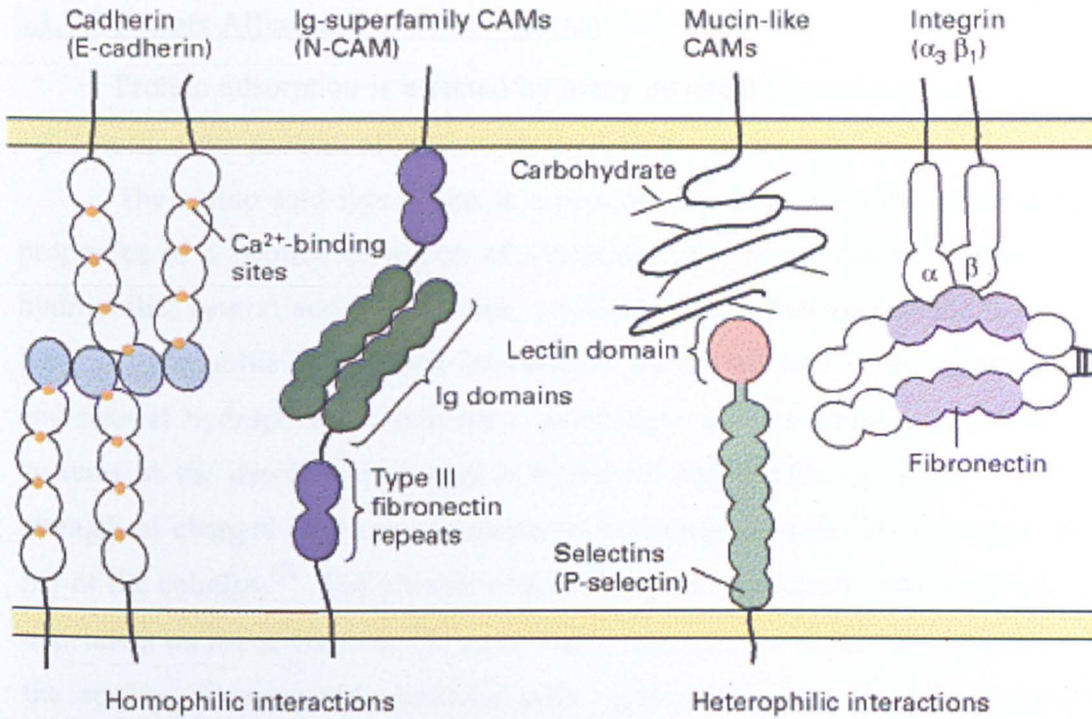


Figure 3-3 Major Families of Cell Adhesion Molecules

Cadherins are trans-membrane proteins that are believed to mediate homophilic cell adhesion by forming a parallel homodimer with another cadherin molecule[133]. The binding of cadherins is Ca^{2+} dependent and the calcium binding ridifies the cadherin molecule and expose residues that form the dimer.

N-CAMs are part of the immunoglobin family of proteins and, like cadherins, promote homophilic cell-cell adhesion. N-CAMs contain various amounts of sialic acid chains which weaken the binding of N-CAMs by electrostatic repulsion so the strength of the adhesion is controlled by differential glycosylation. N-CAMs often possess type III fibronectin repeat sequences that

Selectins mediate leukocyte-vascular interactions to allow white blood cells to move into tissue to protect against bacteria and viruses. The selectins bind to specific sugar sequences and are activated by inflammatory signals that promote the localization of leukocytes at sites of tissue inflammation.[134]

Integrins are composed of an α and a β subunit; there are 17 known α units and 8 known β units and 22 different combinations have been identified in mammalian cells. The binding site of an integrin is comprised of parts of both subunits and is selective towards a particular amino acid sequence.

3.1.4.2 Factors Affecting Protein Adsorption

Protein adsorption is affected by many different factors pertaining to the solid surface, the protein, other proteins present, the solvent and solutes[135].

The amino acid side chains in a peptide sequence affect the electrostatic properties of a peptide or region of a peptide. The region can be neutral and hydrophilic, neutral and hydrophobic, positively charged or negatively charged. Neutral hydrophilic electrostatic interactions are the weakest of the interactions and neutral hydrophobic electrostatic interactions are the strongest in aqueous systems as the desolvation energy is higher for hydrophilic surfaces[136,137]. The strength of charged interactions depend on the charge density, ionic strength and pH of the solution[138]. The amount of a protein that will adsorb onto a surface is dependent on the strength of the electrostatic interaction between the protein and the surface. Consequently proteins with regions where the hydrophilicity or charge is complimentary to the surface will adhere preferentially and in larger quantities to non-complimentary proteins. When a non-complimentary protein has been pre-adsorbed to a surface the addition of a more complimentary protein in solution will result in the displacement of the original protein.

Large proteins have a greater surface area with which to interact with the surface so the amount of larger proteins adsorbed tends to be greater than the amount of a smaller protein[139,140]. The molecular weight and the shape of the protein contribute to the surface area of a protein so the degree of adsorption can be altered by solvent properties that affect the shape of the protein.

The amount of a given protein that adsorbs to a given surface is dependent on the concentration of the protein in solution. Where there are a number of proteins present in solution in varying concentrations then the relative amounts of the proteins adsorbed to the surface will depend on the strength of the electrostatic interactions and the relative concentrations of the components[141].

A single protein can contain regions with each of the different types of electrostatic interaction. Consequently the same protein could adsorb in the same concentration to different surfaces but via different mechanisms and by exposing different areas of the protein sequence. The positioning of the region that preferentially binds to the surface affects the area of the protein that is exposed - the protein can adsorb horizontally along the surface of the material or can stand end on, perpendicular to the surface[142,143]. The exposure of different areas of the

same protein towards cells can affect the cellular response dependent on whether active regions are exposed or masked by the protein conformation. The stability and rigidity of proteins will affect the amount of a protein that will adsorb to a surface as it affects the ability of a protein to adopt a conformation that will preferentially adsorb onto a given surface and the rapidity that a protein can alter confirmation affects the speed of adsorption[144]. However proteins that alter conformation to adsorb strongly onto a surface may lose their bioactive function[145,146].

The aqueous conditions to which a pre-adsorbed protein is subjected to, (such as pH, solutes or competing proteins) can affect the adsorption and / or conformation of the adsorbed protein, which can alter the cell-protein binding through masking or exposing active moieties[16,147].

3.1.4.3 Effects of Protein Adsorption on Cell Behaviour

In general the response of cells to a biomaterial corresponds to the total amount of protein adsorbed onto the surface as the cells cannot adhere to the surface[148-150]. However there are cases in which the cell response is not dependent on the total amount of protein adsorbed as a specific protein can have a different adsorption profile to the majority of the proteins. In these cases a reduced adsorption of the majority of proteins can lead to preferential adsorption of an adhesion protein such as fibronectin that can promote high levels of cell adhesion.

In a series of copolymers of N-isopropylacrylamide (NiPAAm) and N-tert-butylacrylamide (NtBAAm) it was found that total protein adsorption from serum-containing media decreased with increasing NtBAAm concentration (i.e. increasing hydrophobicity)[151]. When the adsorption of albumin and fibronectin were investigated it was found that albumin followed the same adsorption trend as the total protein adsorption but the amount of adsorbed fibronectin increased with increasing NtBAAm concentration. The amounts of cells on the polymers also increased with increasing NtBAAm showing that the adhesion of cells was more strongly affected by the adsorption of specific adhesion proteins that by the total amount of adsorbed proteins.

SAMs with different end groups were formed and it was found that hydrophilic OH and PEG surfaces adsorbed less proteins than amine, acid or

methyl surfaces[136]. However despite the methyl surfaces adsorbing similar amounts of protein to the amine and acid surfaces the percentage of cell attachment and the average cell area was much poorer. It appears that the more hydrophobic proteins that adsorbed to the methyl surfaces were not as successful at promoting cell adhesion as the charged proteins that preferentially adhered to the acid and amine surfaces.

Cells were grown on laminin coated surfaces and it was found that the cell number was dependent on the amount of CM6 epitope available (as assessed by ELISA)[152]. The amount of CM6 available was partly dependent on the total amount of laminin adsorbed on the surface but was also affected by the coating conditions, which were performed in PBS, phosphate-high TWEEN or phosphate-low TWEEN. Whilst the total amount of laminin adsorbed at a given coating concentration was similar with all coating buffers the phosphate-high TWEEN promoted greater availability of CM6 and consequently promoted greater levels of cell adhesion.

Fibronectin was adsorbed to SAMs with OH, acid, amine or methyl functionalities at a fixed density[153]. It was shown that the integrin $\alpha_5\beta_1$ binding efficiency to fibronectin went in the order OH > acid = amine >methyl whereas the integrin $\alpha_v\beta_3$ binding efficiency went in the order acid > amine >> OH = methyl. This shows that the underlying surface chemistry affects the orientation and conformation of an adsorbed protein such that its affinity for different integrins can be substantially altered. The differentiation of cells on these fibronectin coated surfaces was also dependent on the underlying surface chemistry[154].

Serum proteins were adsorbed to colloidal silica surfaces and the total amount of protein was found to be similar on all surfaces[155]. However the cell adhesion was much higher on the control silica that the colloidal silica surfaces implying that the active moieties were less exposed when adsorbed onto the rougher colloidal surfaces.

Adsorbed proteins do not always have a positive effect on cell adhesion – some of the proteins that can adsorb to a surface have a regulatory role in preventing cell adhesion[156].

On the Design, Analysis and Effectiveness of RGD-containing Biomaterials: Literature Review

What a Biomaterials Designer Should Know About RGD

It is well known that the peptide sequence Arginine-Glycine-Aspartic Acid (RGD) can promote cell adhesion through integrin mediated attachment when immobilised in or on a solid material[157-160].

This literature review aims to analyse the effectiveness of RGD-containing biomaterials designed for the purposes of tissue engineering and assess their performance and suitability. It seeks to answer what effect the design of an RGD-containing material will have on the various behaviours of cells. This will be done by investigation of the ways in which RGD peptides can be included in biomaterials to present them towards anchorage-dependant cells. It will also determine how the fabrication of the biomaterial and the design of the cell-based experimental analysis can affect the reported results.

3.2 Materials Design and Synthesis

In the cases under review where materials have been designed to include an RGD-peptide the goals tend to be to promote cell adhesion through the peptide onto materials whose other characteristics (chemical, physical or biological) are desirable. Due to the range of uses that these materials have been intended for these characteristics can vary significantly.

There are many different ways in which an RGD motif can be presented towards cells and the manner in which the peptide is immobilised on a surface can affect the peptide concentration, density, retention, accessibility, arrangement, conformation and activity. This review of synthesis methods therefore concentrates on the manner in which the final biomaterial is assembled and not the ways in which the peptide can be synthesised or derivatised with active functional groups. For this reason the synthesis methods deal with the peptide-containing species (PCS), which have the general formula A-B-(RGD)-B-A where B denotes any number of flanking amino acids and A denotes other chemical groups or species. Peptide-containing species do not always contain all of these groups and include simple peptides (of any length), peptides derivatised

with active functional groups (vinyl groups, bromide etc) and polymers derivatised with RGD (e.g. PEG-RGD).

The methods of presenting RGD moieties towards cells have been divided into 2 groups, physical methods and chemical methods, as the method of including the PCS may affect the properties of the materials. Physical methods of including RGD involve the deposition, adsorption or precipitation of a PCS onto or into a solid material; the PCS is not covalently linked to the material that provides the bulk properties of the system. Chemical methods of presenting RGD towards cells involve the formation of covalent bonds between the PCS and the substrate either as the solid is being formed or by immobilising the PCS onto a pre-formed solid (modification).

Physical Methods:

Coatings - the adsorption of a PCS onto a pre-formed solid material.

Blends - the adsorption or co-precipitation of a PCS and other chemical entities onto a solid or formed into a bulk substrate.

Self-Assembled Monolayers (SAMs)- the adsorption of a PCS, possibly with other chemical entities, onto a pre-formed solid material where the orientations of the chemical species are controlled.

Chemical Methods

Modification - the chemical attachment of a PCS onto a pre-formed surface.

Incorporation - the chemical attachment of a PCS into a solid as it is formed.

3.2.1 Physical Methods

Physical methods are those in which PCS are adsorbed or precipitated onto a surface. The peptide is immobilised by physical forces such as electrostatic attraction or entanglement of polymer chains. The accessibility of the immobilised peptides towards ligands is dependent on the way that coatings or deposits form on the surface and the steric hindrance from other chemical groups.

3.2.1.1 Coatings

Coatings are made by the simple adsorption of PCS or proteins onto surfaces that are pre-formed. The surfaces on which the coating is formed may be 2-dimensional flat sheets or 3-dimensional porous networks[24]. Peptide-containing species of vastly different sizes have been utilised as coatings from the minimal tripeptide sequence[57] to entire proteins containing[93,161] or grafted with[42,43,53] the active sequence. The amount of RGD peptides coated onto surfaces can be controlled (within limits) by the concentration of the coating solution. The coating of a substrate is usually performed in an aqueous buffer at temperatures ranging from 4 to 37°C for times ranging from 30 minutes to 24 hours. The amount of functional moieties present can be assessed by their activities towards antibodies and can be used to denote relative (not absolute) quantities of RGD moieties in proteins or peptides[44,162]. Elloumi et al showed that the adsorbed concentration of a PCS relative to the coating solution concentration up to 10nM was roughly linear but after 10nM a plateau of maximum adsorbed protein was reached[162]. The extent of the coating and the retention of the coating may be affected by the peptide sequence, the chemical groups attached to the peptide (if any) and the substrate on which the peptide is coated. In addition the availability of the peptide sequence to bind to integrins may be affected by the spatial arrangement of the peptide coating on the substrate[163].

A strong anchoring of RGD peptides to surfaces is required and small RGD peptides are often ineffective when coated onto materials if the motif is not firmly attached. This can lead to reductions in the levels of observed cell behaviour[82] as the peptides can become detached and provide soluble competition for integrins (see section 4.4.1.1 Soluble Peptides) or lead to cell death through apoptosis[164].

Sharma et al showed that when a peptide was conjugated to albumin nanocarriers and these were adsorbed to a surface they presented a greater number of active moieties than the simple adsorbed peptide[44].

Sawyer et al showed that a peptide sequence with a polyglutamate chain was adsorbed in significantly greater concentrations than the peptide without the polyglutamate chain[165]. If the concentration of the adsorbed peptide had not been quantified it might have been assumed that the polyglutamate chain was

responsible for the increased cell adhesion rather than the increased concentration of the peptide present. Kurihara et al coated peptides containing 2, 21 or 43 repeat sequences of RGD and found that the amount of $(RGD)_{43}$ coated was 4-fold greater than the other peptides[89] although this may be due to the formation of a self-assembled peptide hydrogel at the surface of the material[166].

However, Rapuano et al showed that H_2N-ENGEPRGDNY-CO_2H and H_2N-YESENGEPRGDNYRAYEDEYSYFKG-CO_2H formed coatings where the amounts of the two peptides deposited were nearly identical over a large concentration range[90].

Bhadriraju et al found that a 2.3kD RGD containing peptide (P2) promoted different levels of cell attachment when coated onto different types of plastic. As the concentration of peptide was not analyzed it is not known whether this is a result of altered adsorption or conformation[93]. Other peptide coatings investigated in the same study as above did not alter the cell morphology or DNA synthesis when the background substrate was altered. In addition there are other studies where coatings have had the same effect regardless of the substrate on which they are coated[53]. However this is a phenomenon that researchers should be aware of.

It should also be noted that coatings may become de-adsorbed from surfaces over time and in competition with proteins in solution, with the de-adsorption being a function of the nature of the PCS and the substrate. The de-adsorption of a peptide from a material was demonstrated by Sawyer et al who investigated the amounts of various coated peptides remaining on hydroxyapatite after agitation in serum-free for up to 48 hours[165]. This study found levels of peptide were significantly reduced during the time periods measured.

It is evident that the extent of the coating, the retention of the coating, the accessibility of the ligand and the conformation of the peptide depend both on the nature of the PCS and the nature of the substrate on which the coating is formed.

3.2.1.2 Blends and Precipitates

Blends are formed by physically combining a PCS and other chemical entities into a solid or by co-depositing them onto a surface in a manner similar to coatings. The formation of the blend can be achieved by co-adsorption onto a

surface[91], pre-adsorption onto a solid prior to processing[167], precipitation (or evaporation) from solution[78,79,168], entanglement of polymer chains[55] and gelation of thermally controlled polymers[32,33,75].

Blends can take the form of a surface modification where the mix of PCS and other species forms a coating on the surface or a bulk incorporation where the entire 3-dimensional solid contains peptide. The coatings formed from adsorption from solution are generally monolayers however coatings formed from precipitation or entanglements are thicker, films of 1-5nm have been reported from spin-coating[79]. Altering the ratio of PCS to other chemical species can control the concentration of RGD in blends. However the orientation and arrangement of the groups cannot be controlled and the peptide may be masked by the other species in the system.

3.2.1.3 Self-Assembling Systems

There are two main types of self-assembly that have been utilised in the production of RGD-containing biomaterials: the self-assembly of amphiphiles at an interface or the spatially controlled adsorption of thiols onto gold surfaces.

Self-assembling systems are created when the interactions between hydrophobic sections and hydrophilic sections drive the assembly of well-defined structures, usually as the formation of monolayers at interfaces. The PCS can be blended with non-peptide containing amphiphiles to control the concentration of peptide in the monolayer. Consequently the amount of RGD present is usually expressed as a molar percentage of the total composition of the monolayer[110,169-171].

Self-assembling systems do not encounter many of the problems encountered with other methods in estimating peptide density as the peptide cannot be obscured by other chemical species as long as the monolayer is formed properly. However this must be proven to be so as some authors have found that some RGD-containing amphiphiles did not pack well at high molar percentages[169,172]. In addition when the monolayers are created by adsorption from solution the ratio of components deposited in a mixed system may not be the same as the ratio of components in solution[171].

3.2.2 Chemical Methods 1: Modification

All modification methods require a functional group in the material to which the peptide can be attached, either via a coupling reagent or a cross-linker. Functional groups that can be utilized include carboxylic acids, amines, aldehydes, alcohols and thiols. There are many different ways of incorporating or modifying a surface[173] to include functional groups but these are outside the scope of this review. These groups can be reacted directly with functional groups on or attached to the PCS. Coupling reagents are used to activate the functional groups on either the material or the PCS and mediate bond formation. The bond formation may be achieved in a one-step synthesis where the peptide and coupling reagent are added simultaneously or in a two-step synthesis where a semi-stable activated group is prepared in the first step and the peptide is added in the second step. Alternatively the functional groups in the material can be reacted with one side of a bifunctional entity, the other side of which reacts with the PCS. These bifunctional chemicals are called cross-linkers as they are commonly used to cross-link proteins and are often hetero-bifunctional (contain two different functional groups)[174].

The approximate RGD concentration in the final material can be controlled by the input concentration. However the coupling efficiency of the synthesis method may not be constant at varying concentrations of peptide leading to a non-linear relationship between input concentration and final concentration. This has been shown by Reinhart-King et al[175] with an NHS activation scheme and Bruers et al[66] with benzoquinone activation. Examples of studies where a linear relationship between input peptide concentration and obtained peptide concentration include [46,124]

3.2.2.1 Coupling Reagents

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is a coupling reagent that mediates the formation of amide bonds between a carboxylic acid and a primary amine. The mechanism of EDC mediated coupling involves the activation of the carboxylic acid followed by the replacement of the EDC with the primary amine.

When coupling RGD derivatives to surfaces there are multiple carboxylic acids and amines present and the peptides will react with each other to form oligo

peptides. In addition peptides possess reactive side chains that will be linked to other chemical species and destroy the integrin binding activity of the peptide particularly with the peptide sequence RGD that contains a carboxylic acid side chain on D and an amine on R. Another problem with EDC coupling is that the activated carboxylic acid can be deactivated by hydrolysis.

Despite this EDC coupling has been frequently used to derivatise surfaces with RGD peptides. Amines[67,126-128,176] or carboxylic acids[64,76] on a material can be utilised to modify a surface with EDC as the coupling reagent as the peptide possess the respective functionality. The solid surface to be derivatised is immersed in a solution containing the peptide, solvent and often N-ethyl morpholine. Dimethyl formamide (DMF) can used as a solvent as the water can be removed from the system and the reaction performed under an inert atmosphere thus removing the problem of hydrolysis[126,127,176]. Alternatively an aqueous buffer such as MES[67] or PBS can be utilised[76]. Coupling times range from 30 minutes[76] to 24 hours[126] and input concentrations of the RGD peptide ranging from 50μM[67] to 2mM[76] have been utilized although many of the references cited do not specify the volume of solution used.

N-hydroxysuccinimide (NHS) or sulfo-N-hydroxysuccinimide (sulfo-NHS) can be added to the reaction mixture to increase the efficiency of an EDC mediated coupling by providing an active intermediate that is not subject to hydrolysis[46,59,62,177]. Rowley et al investigated the effects of changing the EDC concentration, pH, sodium chloride molarity and peptide concentration on the efficiency of an EDC/sulfo-NHS mediated coupling reaction between GRGDY and alginate[46]. It was found that the maximum coupling efficiency was obtained with an EDC concentration that gave 10% theoretical uronic acid activation. The optimum pH was 6.5 and the optimum sodium chloride molarity was 0.3M. A linear relationship between the input amount of RGD and the immobilised density was obtained.

3.2.2.2 Activation Strategies

Activation agents mediate the formation of bonds between substrates and a peptide by a two step reaction. The first step involves the formation of a semi-stable conjugate that activates a functional group on the substrate and the second step involves the replacement of the activating group by the peptide to form the

final product. As the peptide is never present at the same stage as a coupling reagent oligo peptides cannot be formed by activation procedures.

EDC can also be used as an activation agent with the attachment of a peptide sequence being performed in a second step rather than a one-pot synthesis[54,178]. Dicyclohexylcarbodiimide (DCC), a non-water soluble analogue of EDC, can also be used in this manner[179].

N-hydroxysuccinimide (NHS) and its charged, water soluble derivative sulfo-N-hydroxysuccinimide (sulfo-NHS) are frequently used to synthesise PCS and to immobilise peptides on solid materials[19,40,46,68,78,79,81,95,101-105,175]. Carboxylic acids present on a solid substrate are reacted with NHS or sulfo-NHS by means of a coupling reagent, either EDC or DCC[78,79] or by use of N,N,N',N'-tetramethyl(succinimido)uranium tetrafluoroborate (TsU), which forms NHS esters without the need for an additional coupling reagent. The NHS activated esters have a half life of minutes to hours at neutral pH and can be stored for months if dried. NHS activated esters containing vinyl groups are also stable enough to be copolymerised into polymers or cross-linked gels[68,180]. The structures of some reagents used to form NHS activated esters are shown in Figure 3-4 below.

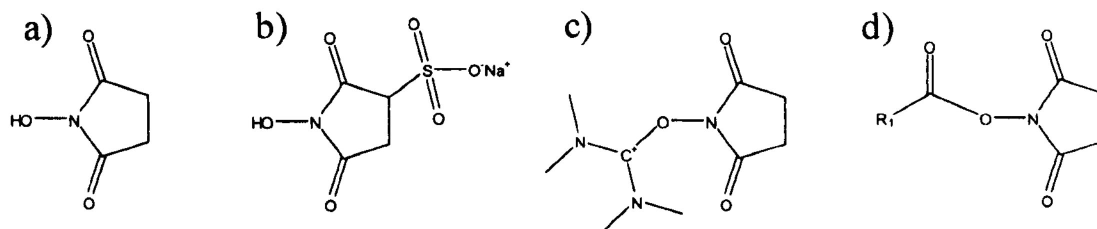
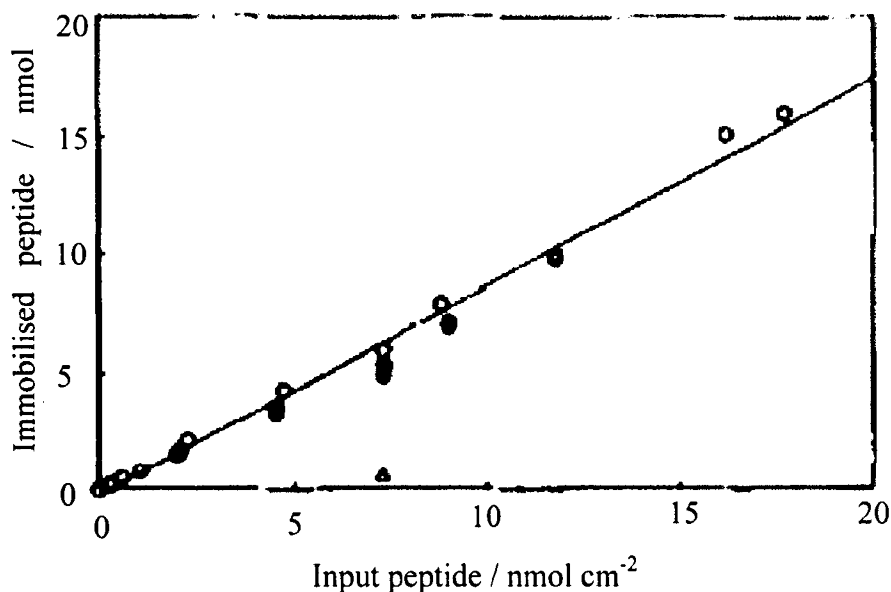


Figure 3-4 Structures of a.) N-hydroxysuccinimide, b.) sulfo-N-hydroxysuccinimide, c.) TsU and d.) succinimide activated carboxylic acid.

Reaction of the peptide to an NHS activated ester occurs through free amines in the peptide to form amide bonds and can be performed in aqueous media or a combination of aqueous media and organic solvents[40,79]. Amine functionalities on side chains can also react with the NHS activated esters. The peptide concentration immobilised onto the substrate may be controlled by the amount of carboxylic acids incorporated into the system or by the input amount of peptide reacted with the system.

Brandley et al and Reinhart-King et al both investigated the relationship between input concentration of YAVTGRGDS and the amount of immobilised peptide onto acrylamide based gels copolymerised with the succinimide ester of acrylamidohexanoic acid[68,175]. Brandley et al found a linear relationship between input concentration and amount of immobilized peptide over a concentration range of 1-20nmol/cm^2 that was not dependent on the amount of NHS activated ester incorporated into the system. Reinhart-King et al reported a non-linear relationship over the concentration range 0.001-1mg/ml (~1.2nmol/cm^2 to 1.2μmol/cm^2). The relationships between input concentration and immbobilised peptide concentration found by the two sets of authors are shown in Figure 3-5 overleaf.



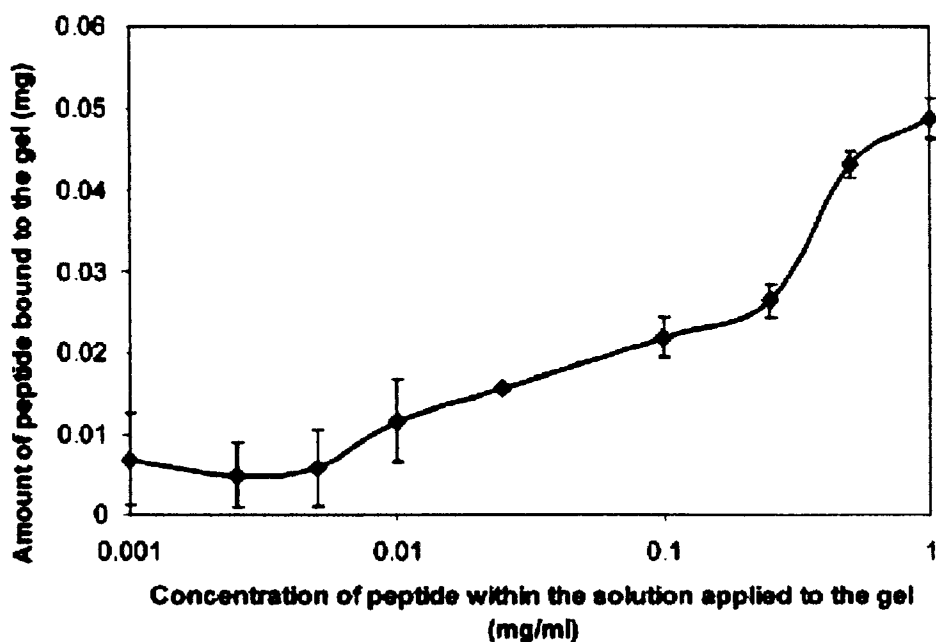


Figure 3-5 Top - Efficiency of YAVTGRGDS immobilisation to acrylamide-co-bisacrylamide gels containing 0.1 (open triangle), 1 (closed circle) and 10 (open circle) μmol/ml of succinimide ester activated acrylamidohexanoic acid. Reaction performed in 50mM HEPES (pH 8) for 16 hours at 4°C. Reproduced from Brandley et al[†,68].
Bottom- Efficiency of YAVTGRGDS coupling to N-hydroxyl succinimide activated acrylamidohexanoic acid (20μmol/ml) copolymerised with N,N'-bisacrylamide (5 wt%) gels in 50mM HEPES buffer (pH8) at 4°C for 2 hours. Reproduced from Reinhart-King et al[‡,175]

† Reproduction of Figure 1 from "Brandley, B. K. & Schnaar, R. L. Covalent Attachment of an Arg-Gly-Asp Sequence Peptide to Derivatizable Polyacrylamide Surfaces: Support of Fibroblast Adhesion and Long-Term Growth. *Analytical Biochemistry* **172**, 270-278 (1988)" with permission from Elsevier Ltd.

‡ Reprinted with permission from "Reinhart-King, C. A., Dembo, M. & Hammer, D. A. Endothelial Cell Traction Forces on RGD-Derivatized Polyacrylamide Substrate. *Langmuir* **19**, 1573-1579 (2003)" Copyright 2003 American Chemical Society

The reason for the non-linear relationship found by Reinhart-King may be due to the increased concentrations or it may be due to the short coupling time utilised (2hrs at 4°C). Drumheller et al showed that the coupling of YRGDS to succinimide polymers at high concentrations took up to 12 hours to complete although the solvent utilised in this case was DMF:DIPEA:H_2O 98.5:0.5:1rather than aqueous buffer[40].

Alcohols can be activated with mesyl or tresyl chloride to form an intermediate that reacts with amines from a peptide to form new C-N bonds[63,107,129,130,181-184]. The mechanism for this reaction is shown below in Figure 3-6.

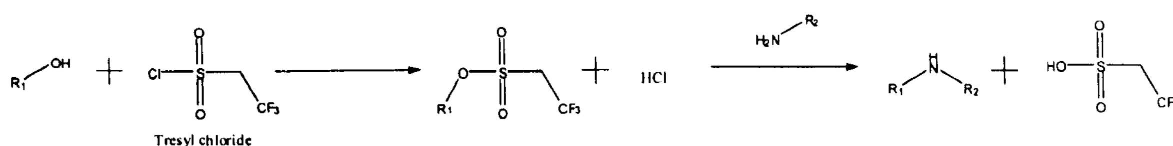


Figure 3-6 Reaction scheme for activation of alcohols by tresyl chloride followed by replacement with primary amines.

Alcohols can also be activated with benzoquinone, which attaches to the substrate to form an ether and provides an additional site that is reactive towards amines.

Bruers et al investigated the pH and concentration dependence of the reaction of GRGDS with benzoquinone activated surfaces[66]. Maximum peptide immobilisation was achieved at pH 3 and the efficiency of the coupling decreased with increasing input peptide concentration. Benzoquinone activation has also been utilised to immobilise peptides with diene functionality via a Diels-Alder reaction[185].

Aldehydes can be utilised to immobilise peptides onto surfaces via primary amines, which act as nucleophiles and form imines[186,187] that can be reduced to the more stable secondary amines. Sodium cyanoborohydride has been utilised as a reducing agent[48,188,189]. The coupling efficiency of reductive amination was investigated by Glass et al who found an exponential relationship between the amount of input peptide and the amount of bound peptide[186].

Thiol moieties in keratin[52] or BSA[190] have been used to immobilise peptides containing bromine residues as the sulphur replaces the bromine in an S_N2 reaction to form a sulphide. More normally with thiol-based immobilisation chemistry a peptide sequence containing cysteine is utilised to immobilise a peptide onto a surface via the thiol side chain of cysteine. A Michael-type

addition reaction can be utilised to immobilise thiols to surfaces containing α,β-unsaturated carbonyl groups such as methacrylates[111] or maleimides[191]. An example of a Michael-type addition reaction is demonstration in Figure 3-7 below.

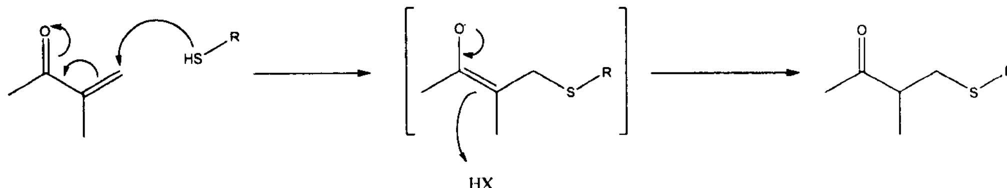


Figure 3-7 Example of a Michael-type addition reaction of a thiol to a methacrylate.

Alternatively the thiol can be utilised to form disulphide bonds with a thiol activated by DMSO[51] or by replacing activated disulphide moieties[114,192,193] on the surface of the substrate.

Aryl azides form nitrene groups when exposed to UV-light that can initiate addition reactions with double bonds, insert into C-H and N-H sites or undergo a ring expansion to react as a nucleophile with a primary amine[194]. The latter reaction path dominates when primary amines are present in the sample. Phenyl azides can be incorporated into PCS and utilised to immobilise the peptide onto a solid surface[58].

Benzophenone can also be used as a photoactive strategy to immobilise peptides onto surfaces. The amount of peptide incorporated is proportional to the exposure of UV-irradiation as shown by Herbert et al[195].

3.2.2.3 Bifunctional Cross-Linkers

There are a large variety of commercially available cross-linkers, which makes them attractive prospects for functionalising surfaces with peptide sequences. In addition the cross-linkers contain a spacer arm that may be useful to distance a peptide from the bulk of the substrate.

Homo-bifunctional cross-linkers consist of molecules that have been derivatised with two of the same active functional group. Disuccinimidyl cross-linkers are utilised to derivatise surfaces containing amine functionalities with peptides via an amine in the peptide. Different spacers can be utilised to form the cross-linker, disuccinimidyl suberate[54] and its sulfonated analogue bis(sulfo-succinimidyl) suberate contain a $(CH_2)_6$ hydrophobic spacer between the two NHS activated esters. Ethylene glycol bis(succinimidyl succinate)[18,25] and its

sulfonated analogue[26] contain a hydrophilic spacer between the two activated end groups. The structures of some disuccinimidyl crosslinkers are shown in Figure 3-8 below.

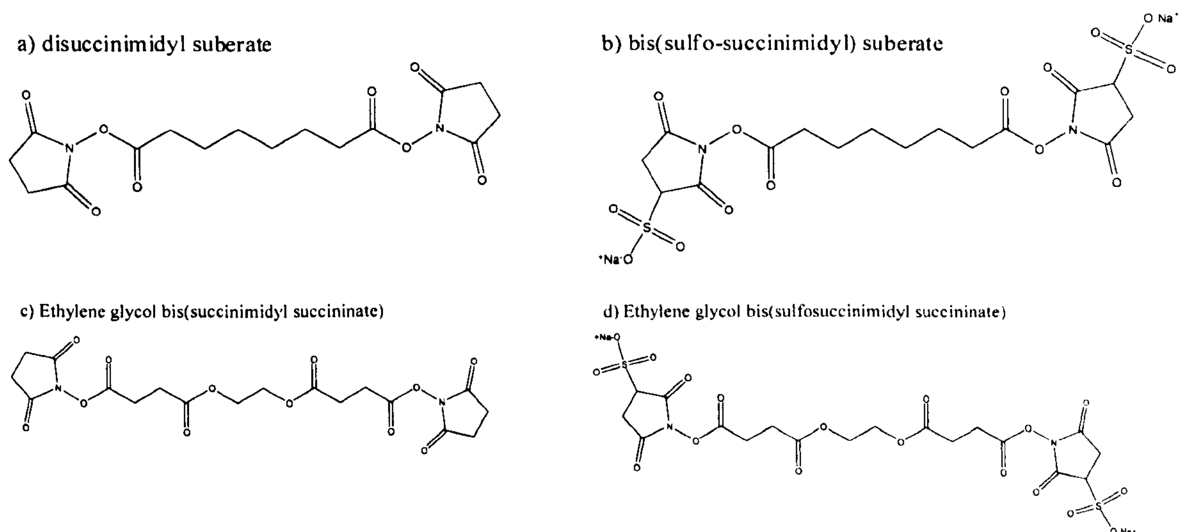


Figure 3-8 Disuccinimidyl homo-bifunctional cross-linkers.

Hetero-bifunctional cross-linkers contain two different functional groups activated to react with different functionalities in the substrate and peptide. Most of the hetero-bifunctional cross-linkers contain a succinimide activated ester that reacts with amines on the peptide or substrate.

N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate (LC-SPDP) contain a succinimide activated ester to react with amines on the surface of the substrate and an activated disulphide bond that is displaced by the SH functional side chain of cysteine[42,44,196-198]. The LC-SPDP contains a longer spacer arm that distances the peptide from bulk. Both derivatives are also available as their sulfonated analogues and these have also been utilised to attach RGD peptides to surfaces[199]. The structures of succinimidyl and dithio based crosslinkers are shown in Figure 3-9 below.

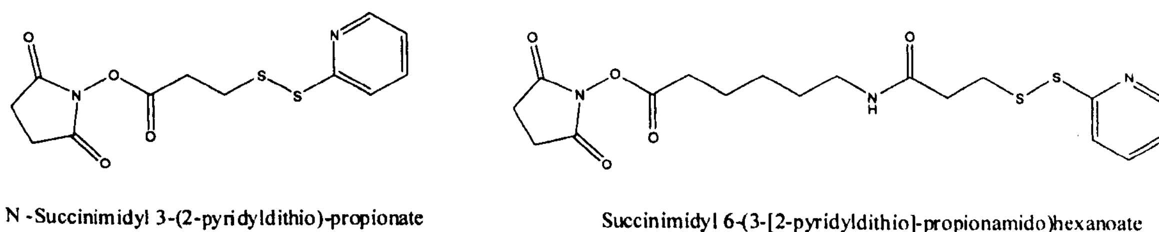


Figure 3-9 Succinimidyl and dithio based hetero bifunctional crosslinkers.

N-succinimidyl-3-maleimidopropionate (SMP)[200,201], N-succinimidyl trans-4-(maleimidylmethyl)cylcohexane-1-carboxylate (SMCC) and N-succinimidyl-4p-maleimidophenyl-butirate (SMPB) and their sulfonated analogues[43,74,80,120,121,124] also react with thiols from cysteine but in a Michael-type addition reaction as discussed in section 1.2.3.3. Structures of crosslinkers with succinimidyl activated ester and reactive groups for Michael-type addition reactions are shown in Figure 3-10 below.

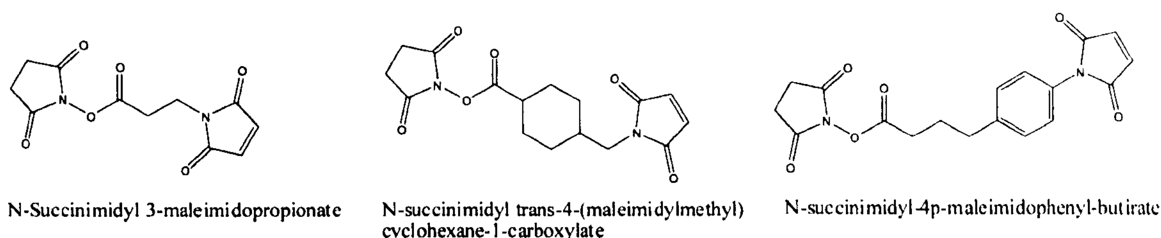


Figure 3-10 Hetero bifunctional cross-linkers with a succinimidyl activated ester and a reactive group for Michael-type addition reactions.

N-Succinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate (SANPAH) contains an NHS activated ester that reacts with free amines in a peptide and a phenylazide photo reactive group that can utilised to immobilise the peptides onto a surface[50,106].

FNIII$_{7-10}$ was attached to BSA coatings using various homo-bifunctional or hetero-bifunctional linkers and the relative amounts of accessible ligands were assessed by ELISA[202]. The cross-linkers investigated were dimethyl 3,30-dithiobispropionimidate (DTBP), 3,30-dithiobis[sulfo-succinimidyl propionate] (DTSSP), N-[ε-maleimidocaproyloxy]sulfosuccinimide ester (sulfo-EMCS), sulfosuccinimidyl-4-(P-maleimidophenyl) butyrate (sulfo-SMPB), N-[κ-maleimidoundecanoyloxy] sulfosuccinimide ester (sulfo-KMUS) and sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido] hexanoate (sulfo-LC-SPDP). The ELISA determined amount of FNIII$_{7-10}$ for coating concentrations of 20μg/ml of each cross-linker divided by the background levels of protein were found and designated as the coupling indexes. It was found that the homo-bifunctional amine reactive cross-linkers (DTSSP) and dimethyl 3,30-dithiobispropionimidate (DTBP) produced surfaces with much greater concentrations of accessible peptides than the other cross-linkers. The coupling index determined to be 83 and 44 for DTBP and DTSSP respectively whereas the coupling index for the other crosslinkers was found to be 8.2 or lower. The

coupling index as determined by ELISA probably refers to a combination of peptide immobilisation and peptide accessibility.

3.2.2.4 Distribution of Surface-Modified RGD

The majority of modification procedures are carried out with non-protected peptides. In some cases, it appears that the amino acid side chains of RGD will react with the activated substrate or other peptide molecules. There is a general convention to assume that the determined amount of peptide is evenly distributed over the surface of the substrate whereas in fact the peptide may in fact have reacted to form oligo-peptides and be localised into clusters of repeat sequences (see section 4.2.2.1). This clustering usually occurs when coupling reagents are utilised and the peptide is present at the same time as activating agents. The clustering of RGD peptides on a surface through a modification procedure was observed by Senyah et al[119]. The clusters can be seen through confocal laser microscopy and scanning electron microscopy in Figure 3-11 overleaf.

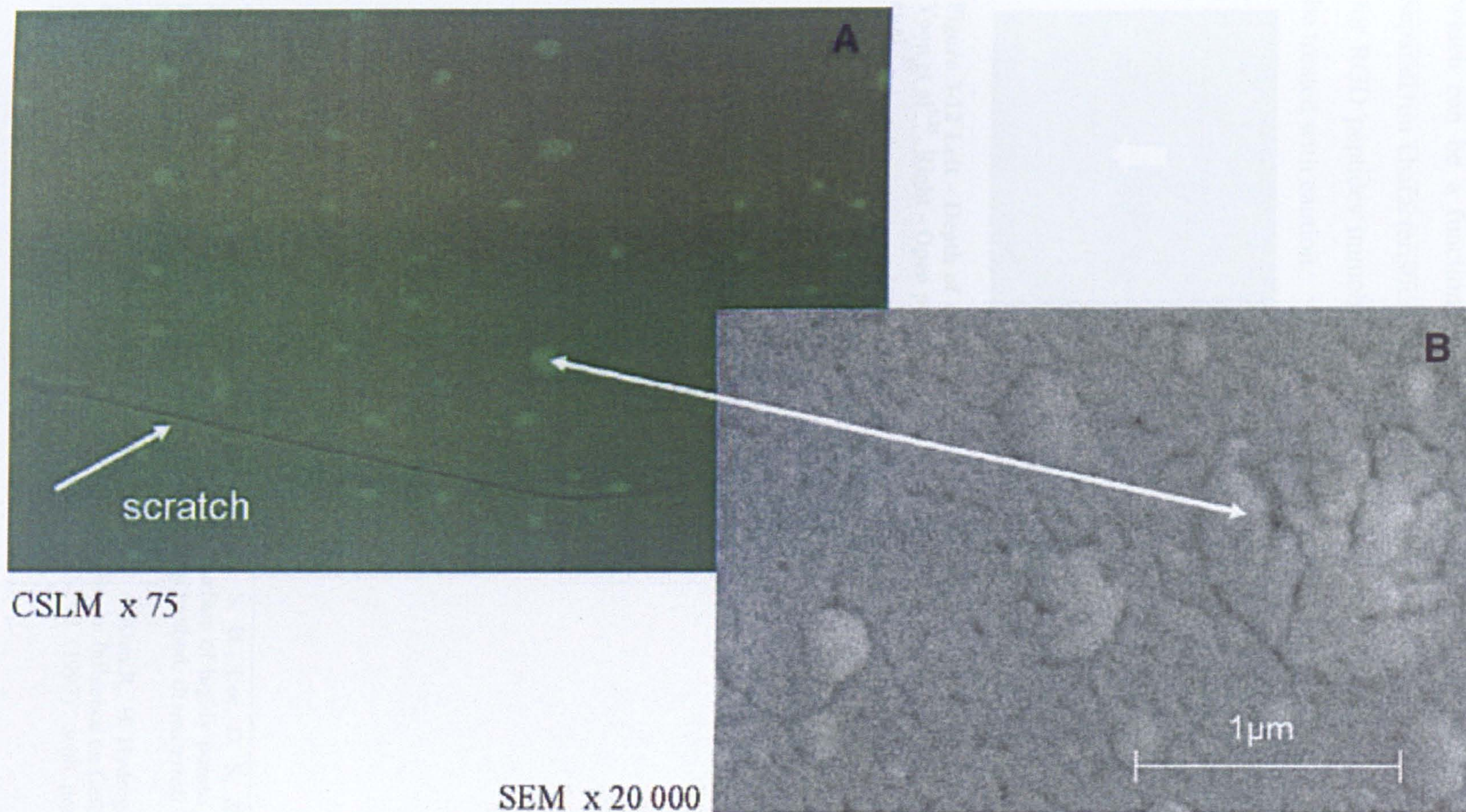


Figure 3-11 Immobilisation of fluorescent RGD-peptide onto glass, observed by A) confocal laser scanning microscopy and B) scanning electron microscopy. Reproduced from Senyah et al[†,119]

† Reproduction of Figure 1 from "Senyah, N., Hildebrand, G. & Liefeith, K. Comparison between RGD-peptide-modified titanium and borosilicate surfaces. *Analytical & Bioanalytical Chemsitry* **383**, 758-762 (2005)" with kind permission of Springer Science and Business Media

Whilst materials like glass and metals do not allow the access of peptides and reagents into the bulk of the material many polymers are made as cross-linked gels or blends and they contain pores[25] as shown in Figure 3-12. The depth of the surface modification will therefore depend on the size of the pores, which can be a function of the concentration of monomers[203] or the phase-separati†on characteristics[85]. Consequently many of the reported concentrations for RGD peptides immobilised onto polymers by modification methods should be treated with caution.

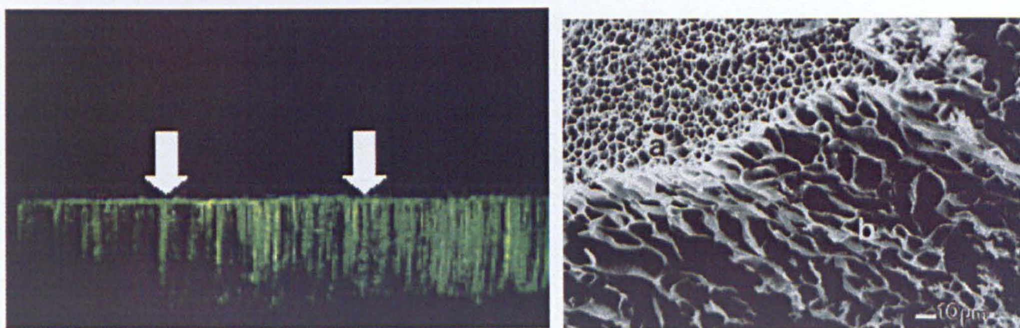


Figure 3-12 Left - Depth of surface modification to PLGA blend films. Reproduced from Yoon et al[†,25]. Right - Open porous structure of a HMPA hydrogel. Reproduced from Plant et al[‡,85].

† Reproduction of Figure 3 from "Yoon, J. J., Song, S. H., Lee, D. S. & Park, T. G. Immobilization of cell adhesive RGD peptide onto the surface of highly porous biodegradable polymer scaffolds fabricated by a gas foaming/salt leaching method, *Biomaterials* **25**, 5613-5620 (2004)" with permission from Elsevier Ltd.

‡ Reproduction of Figure 2 from "Plant, G. W., Woerly, S. & Alan R., H. Hydrogels Containing Peptide or Aminosugar Sequences Implanted into the Rat Brain: Influence on Cellular Migration and Axonal Growth. *Experimental Neurology* **143**, 287-299 (1997)" with permission from Elsevier Ltd.

3.2.2.5 Comparison of Strategies

The coupling efficiency of a peptide to a substrate is affected by the peptide, the material, coupling reagents and the conditions under which the coupling is performed. If the material on which a peptide is being immobilised is altered the coupling efficiency may also be altered as shown by Yamaoka et al who derivatised poly(L-lactides) containing various β-alkyl α-malate-units with RGD under the same conditions[179]. The amounts of immobilised peptide in μg/cm^2 was analysed and it was found that the alkyl group had a large effect on the amount of peptide. (R = H, 7.7 μg/cm^2; R = CH_2Ph, 3.0 μg/cm^2; R = $C_{12}H_{25}$, 1.7 μg/cm^2; PLLA alone, 0.95 μg/cm^2). This may also have been the case in a situation where N-isopropylacrylamide was copolymerised with different carboxylic acid containing monomers, namely 2-carboxyisopropylacrylamide (CIPAAM) and acrylic acid (AA)[76]. RGDS was reacted with both polymers under identical conditions (2mM RGDS plus 2mM EDC in PBS, pH 7.4, 24 hours, RT). However, those polymers synthesised with CIPAAM were significantly better at promoting cell adhesion and spreading than those synthesised with acrylic acid. As the amount of RGD that had been immobilised was not quantified it is difficult to tell whether this effect is due to a change in RGD concentration or a change in integrin binding induced by the changing conditions.

Bouhadir et al investigated the immobilisation of RGD peptides to sodium poly(guluronate) and poly(aldehyde guluronate) with varying conditions[48]. The same amounts of radio-labelled GRGDY were used without reagents, with carbodiimide/sulfo-NHS coupling and with a reductive amination procedure. Slightly higher amounts of peptide were incorporated via the sodium cyanoborohydride ($NaCNBH_3$) reductive amination.

Different methods of modifying surfaces give different coupling efficiencies and the efficiency can be dependent on the concentration. Patel et al created poly(methyl methacrylate) peptide modified surfaces with identical final chemistries via hydrolysis or aminolysis[80]. At low concentrations (<0.5μM) the relative efficiencies of the two methods were similar. However at higher concentrations the coupling efficiency of the aminolysis method was higher.

Even when the method utilised to derivatise a material with RGD is kept constant the sequence in which the steps are performed may affect the amount of peptide, accessibility, or binding strength. This was shown by Irvine et al who modified comb polymers with a methyl methacrylate backbone and PEO side chains[79]. The combs were modified in solution, activated in solution and coupled in situ, or both activated and coupled in situ. Coupling efficiencies were approximately 30% lower when the combs were activated and coupled in situ in comparison to the other two methods, which is likely to be due to steric hindrance in the activation step.

3.2.3 Chemical Methods 2: Incorporation

Peptides can be incorporated into polymers by including a polymerisable group such as a carbon double bond into a peptide-containing species (PCS) and then copolymerising the PCS with other monomers. Alternatively gels can be produced from proteins or polymers containing active functional groups that can be cross-linked with enzymes or bifunctional cross-linkers such as those detailed in section 1.2.3.3.

3.2.3.1 Chain-growth Polymerisation

The creation of the PCS is often achieved by one of the chemical strategies utilised for modification (see section 1.2.3). Polymerisation is achieved by use of an initiator that can be activated by the use of heat, visible light, UV light or chemical reaction. Examples of polymerisation initiators that have been utilised to form RGD-containing biomaterials include azobisisobutironitrile (heat)[83,84], 2-hydroxy-2-methyl propiophenone (UV)[31], 2,2-dimethyl-2-phenyl-acetophenone (UV)[30,94,98], 2,2-dimethoxy-2-phenyl-acteophenone (UV)[41,112] and ammonium persulfate (chemical)[68-71]. These initiators have been chosen because they are non-toxic towards cells when immobilised in a polymer and / or when in solution so that cells can be encapsulated in polymers as they are formed.

The concentration of the peptide in a polymer can be controlled by altering the ratio of PCS and other monomers. When copolymerised with other monomers the amount of PCS incorporated into the polymer will be dependent on the relative polymerisation rates of the components. Acrylamidohexanoic acid was derivatised with an RGD-containing peptide and copolymerised with acrylamide and bisacrylamide to form cross-linked gels[68]. Using this method

75 ± 2% of the added peptide was incorporated over a concentration range 50-260 nmol/cm^2. The acryl-containing PCS was not purified prior to use so part of the loss of peptide may be due to inefficient coupling of the peptide to acrylamidohexanoic acid prior to copolymerisation. The coupling was performed via NHS chemistry and a 20-fold excess of activated ester was utilised. Other investigations with 25-fold excess of activated esters have reported coupling efficiencies of 84% w.r.t. the peptide[41].

The incorporation of different PCS into PEG-DA hydrogels was investigated by Hern et al who found that acryloyl-YRGDS was incorporated with 51-75% efficiency and that acryloyl-PEG-YGRGDS was incorporated with 81-97% efficiency[41]. This reflects the relative polymerisation rates of the two PCS species investigated. However it should be noted that the PCS were not purified after synthesis and the efficiency of the PCS synthesis was only 84% so that actual peptide concentrations were only 40-81% that of the nominal concentrations. Polymerisation methods can also be utilised to create gradients of peptide within a gel by the use of micro-fluidic commercial gradient makers[31,69].

Biesalski et al polymerised self-assembled amphiphiles at an interface[172]. Polymerisation was achieved via UV irradiation activation of alkyne bonds. The polymerisation of vinyl containing PCS can also be utilised to add RGD functionality to pre-formed polymers by making use of non-polymerised double bonds contained in the material[77]. However it seems likely that the PCS will polymerise to form oligo-peptides and the surface distribution of the peptide will be uneven.

3.2.3.2 Cross-linking

Alginate can be ionically cross-linked by calcium sulphate and this strategy was used to form gels from alginate pre-modified with RGD blended with non-modified alginate[45].

Gelatin can be cross-linked with trans-glutaminase and RGD peptides containing glutamine have been incorporated into gelatin gels via this method[204]. Interestingly when RGD peptides without glutamine were treated by the same system they also promoted cell adhesion and proliferation, although to a lesser extent, indicating that the peptides were probably immobilised in part by physical entrapment as well as cross-linking.

Factor XIIIa can cross-link fibrin and the peptide sequence LNQEQ and this methodology was utilised to form fibrin gels with RGD incorporated through a peptide sequence containing both LNQEQ and RGD[27,35,205]. Maximum peptide incorporation was achieved at 1mM input concentrations and declined at higher concentrations, which was attributed to limited amounts of cross-linking enzyme being available. As the input concentration of peptide increased the amount of free peptide that was not cross-linked into the gel also increased with implications for competition for integrins between immobilized and solubilised peptides.

3.2.4 Assessment of RGD Concentration

In many of the references cited a variety of RGD concentrations are studied in order to optimise the cell response. Altering either the input amount of the peptide containing species (PCS) or the amount of active functionalities present on each PCS can generally control the concentration of RGD. However the actual amount of peptide presented towards cells may depend on a number of other factors such as the incorporated / deposited amount of peptide, the spatial arrangement and the accessibility of the peptide.

3.2.4.1 RGD Concentration: Notations and Implications

In addition there are a number of different forms in which the amount of RGD present can be expressed in terms of the mass or molar amount of the peptide and the surface area, volume, mass or molar amount of the substrate in question. The most appropriate notation for each material is utilised and generally depends on the synthesis method however this means that it is difficult to compare RGD concentrations from different synthesis methods as the units of the concentration or density may be different. Where units have been converted from one type to another assumptions are made such as the depth of a modification procedure or the density of a cross-linked polymer which may not be accurate and may be misleading unless all assumptions and calculations are publicized.

The amount of peptide immobilised on a surface by physical methods and incorporated onto a surface or into a material by chemical methods will depend on the input amount of peptide relative to other species and the efficiency of the immobilisation procedure. Assessment of RGD-concentration should be

performed qualitatively to discover the effects of peptide incorporation at a known surface density on cell behaviour. Non-porous systems such as glass or TCP will present ligands towards cells in a quasi-2D system. In these 2D systems the amount of peptide available for binding is dependent on the concentration of peptide in terms of number of active moieties per unit surface area. The amount of peptide available for binding in 3D systems such as cross-linked gels is dependent on the density of peptide and the depth that integrins can penetrate to access ligands, which is often assumed to be 10nm.

Coatings are usually 2 dimensional as the peptide forms a layer on the surface; the units used reflect this. If the extent of peptide coating has been quantified (see section 1.3) the concentration can be expressed as mass per unit area[42,206], moles per unit area[206,207] or distance between peptide molecules[42,113]. If the coating forms a multi-layer coverage instead of a monolayer or less this notation may no longer be appropriate as there will be a non-accessible RGD content in the bulk. It is worth noting that fibronectin forms a monolayer coating at a concentration of ~350ng/cm^2, equivalent to 0.692pmol/cm^2 of RGD species[170]. Coatings of peptides or peptide containing species onto surfaces are often designated by the amount of peptide in the coating solution expressed as either moles per unit volume[42,53,90,118,162] or weight per unit volume[51] combined with a volume of coating solution per unit area. Where comparisons between different peptide-containing species are being made care must be taken to ensure that the units are converted to moles per unit area to ensure that the numbers of active moieties are comparable. For example a poly (l-lysine-graft-GRGDS) will have significantly less active moieties per mass unit than a short amino acid sequence.

Blends of materials deposited as coatings or processed into a shape often express the amount of RGD present as a function of the amount of non-peptide containing material present. This can be done as a mass per mass (weight %)[78,79,167,168], mass per volume (w/v %)[55], moles per mass, moles per volume or moles per mole (mol %)[110]. Where the peptide amount is measured in mass it must be noted that the mass will be of the entire peptide containing species, which may mean that very different amounts of active moieties are present in systems that purport to have similar concentrations. Where the peptide amount is measured in moles it must be noted that the molar masses of the other

components in the system may have a large effect on the density of the active moieties. Possibly the most relevant notation for this type of system would be moles per effective volume of solid as this notation reveals the peptide density and spacing.

Attachment of PCS to a pre-formed solid is generally assumed to be a surface only modification as many solids, such as glass, do not allow access of reagents into the bulk. The assessment of RGD concentration in materials formed by modification procedures usually assume that the reagent penetration depth is the same as the integrin accessible depth of 10nm and treats the results in a 2D manner. This may not be appropriate where the material being modified is porous as shown in section 3.1.3. It should also be noted that the majority of the assessment protocols are performed on the materials in a dried state and many polymeric materials may expand in aqueous environments as they adsorb water. The units of concentration for a modified surface are generally moles per unit area and reflect the assumption that the immobilization of the PCS occurs only to a depth of 10nm, which is also assumed to be the depth that integrins can have access to the peptide. However this may not be true in all cases and where the concentration of peptide has been assessed in a 3D material the peptide concentration may be spread over a greater depth of material than the integrins are able to access. Rowley et al estimated the penetration depth of modification reagents to be 50nm[46].

The units of peptide concentration that are utilised for incorporation methods are usually in terms of the total mass of the substrate or the fraction of monomeric components containing peptide. Analysis of the peptide concentration will give an amount of peptide per mass of substrate which can be converted to moles per unit area if assumptions are made as to the depth that cells can perceive ligands, the density and swelling characteristics of the substrate under culture conditions. However in many cases the swelling characteristics of a substrate are not measured and the peptide concentration quoted is usually that of the amount of peptide in the dry weight of material.

The implications of the different notations utilised for RGD concentrations impact heavily on the capacity to make comparisons between different systems containing RGD peptides. In addition many researchers do not carry out analysis of the actual peptide concentration and rely on the assumption

that the amount of active peptide moieties presented towards cells is proportional to the nominal peptide concentration utilised in the synthesis. This leads to further confusion over the peptide concentration required to effect a significant response from cells.

3.2.4.2 Quantification Methods

Radio labelling can be utilised to quantify the amount of RGD present by incorporating ^{125}I, ^{14}C, or ^{35}S into a peptide sequence. ^{125}I is usually incorporated into a peptide sequence by oxidative iodination of tyrosine (Y)[40,41,45,46,48,68,114,121,207,208]. ^{14}C can be incorporated into a peptide sequence by reductive methylation of lysine[171,195] or by reaction of radio-labelled phenylglyoxal with arginine side chains[117]. ^{35}S is incorporated by utilising radio-labelled cysteine in the peptide synthesis[201]. Radio-labelling can also be utilised to estimate the efficiency of peptide incorporation by replacing the peptide in the coupling step with a chemical species that contains the same reactive functional group and is commercially available as a ^{14}C radio-labelled species[190].

Amino acid analysis is a general technique that can be conducted on any material that is capable of being degraded[23,51,64,66,186,209]. The material is hydrolysed, usually with 6M HCl at 110°C and the molar amounts of each amino acid per sample can be determined. The amount of peptide included in a material is calculated from the average molar amount of each amino acid divided by the mass of the sample which gives a peptide concentration in units of mol g^{-1}. Where the material being derivatised with RGD is a protein the background substrate will contain amino acids and the amount of RGD-containing peptide incorporated can be determined as the increase in amounts of the amino acids that are included in the PCS. The analysis of RGD concentration after degradation of a substrate can also be performed using the ninhydrin assay for amines by degrading the substrate by acid[179] or base hydrolysis[26,86].

The incorporation of a dye or fluorescent into a peptide sequence can be used to quantify the amount of immobilised peptide[79,80,165,167,210] and visualise the localisation of the peptide[119]. Assays for specific functional groups can be utilised to determine the RGD concentration from the Sakaguchi assay for guanidine groups on the arginine side chain[211] or Ellmans reagent for thiols[96]. Methods of analysing the amount of RGD in solution from either the coupling

solution or substrate degradation products include HPLC[50,73,128,181], UV spectroscopy[175], gel permeation chromatography[205], NMR[75,212,213] and size exclusion chromatography[27,35]. The efficiency of a coupling reaction can be assessed indirectly by the quantification of peptide concentration in the reaction solution before and after coupling however this may lead to reduced reliability. Semi-quantitative assays for the amounts of RGD present can be performed using ELISA to determine the binding of antibodies to immobilised ligands[44,89,166,214]. This procedure has the advantage that it quantifies the amounts of accessible ligands but the disadvantage that the assay only shows the relative amounts of peptide not the actual concentration.

Spectroscopic ellipsometry analysis can be utilised to determine the thickness and the molar refractivity index of coatings or modification procedures and can be used to calculate the surface density of immobilised species[124,206]. The mass of adsorbed peptide containing species can be used to determine the RGD concentration if the moles of peptide per mass unit are already known. The mass of adsorbed coatings can be determined using optical waveguide lightmode spectroscopy (OWLS)[113]. It should be noted that the majority of the assessment protocols are performed on the materials in a dried state and many polymeric materials may expand in aqueous environments as they adsorb water.

3.3 Assessment Methods and Their Effect on Outcomes

After an RGD-containing material is synthesised it must be assessed to determine whether it is fit for purpose. These biomaterials are generally utilized for two main purposes; investigation into cellular behaviour or for use in tissue engineering. The majority of the research is performed on cells cultured on the material *in vitro* either as an investigative study or as an initial evaluation method to generate an indication of the suitability of the material for tissue engineering.

The criteria for whether a material is fit for purpose is highly dependent on the intended use and the initial assessment as to its suitability can be measured in a number of different ways. Usually the measure of success of the material is determined by its ability to improve an aspect of cellular behaviour above that of a non-peptide control. The aspects of cellular behaviour that have been used as assessment methods include adhesion (cell spreading, cell number, cytoskeletal organisation), attachment strength, viability, proliferation, motility, secretion

(DNA, RNA, cell specific functions) and differentiation. Note that disparate properties can be desirable dependent on the usage of the material; extracellular matrix deposition can be a desirable feature indicating the successful adhesion and function of cells or it may be an undesirable feature on non-fouling materials as the surface becomes remodelled and allows protein adsorption and non-specific interactions. The outcome of the assessment, in terms of whether the material is deemed successful, may depend on which aspect of cellular behaviour is examined and also the methodology used for evaluation. For example a material may be successful at promoting cell adhesion but not spreading so an assay based method may report an increase in cell number or function whereas a visual based method would show the lack of cell spreading. In addition there are a number of experimental parameters involved in *in vitro* cell culture that may have an effect on the reported outcomes; these include cell type, seeding density, time of analysis, culture conditions and pre-treatment of the material. The aspects of the required cell behaviour, experimental design and analysis methods that may affect reported outcomes are analysed in more detail below.

3.3.1 Cell Behaviour

RGD moieties that bind to integrins can be found in a large number of ECM proteins including fibronectin, vitronectin, fibrinogen, osteopontin, collagen, Von Willebrand factor, laminin and thrombospondin[215]. The RGD sequence in each protein has different affinities for the various integrins produced by cells. Integrins are a family of transmembrane proteins that control various aspects of cell behaviour such as cell adhesion, cytoskeletal organisation and migration. The majority of mammalian anchorage-dependent cells produce integrins capable of binding to RGD peptides. However, the integrin expression profile and the activation of the integrins varies between cell types and the local environment of the cells[216,217]. The binding of the RGD motif to integrins regulates a large number of cell responses of which the best known are the adhesion, spreading and re-organisation of the cytoskeleton to form focal contacts. However the binding of the RGD motif can also regulate cell migration, proliferation and differentiation[218]. Different integrins can produce different cellular responses and have variable affinity for RGD sequences which leads to

the behaviour of cells on peptide-containing surfaces being affected by the nature of the peptide sequence and its micro-environment.

The ability of an RGD-containing surface to promote the different facets of cellular behaviour is likely to depend on the binding affinity of the peptide in its current environment to a selected integrin that regulates that function[219]. For example an RGD peptide may be able to bind to the integrin that regulates adhesion and promote this function but may not be able to bind to the integrin that regulates migration. Consequently when RGD biomaterials are analysed the aspect of cellular behaviour that is examined will have a large effect on whether the material is reported to be fit for purpose.

The assessment of cells cultured on biomaterials will give different results depending on the time after seeding that the analysis is conducted as cell behaviour is time-dependent and different aspects of the behaviour will be analysed at different time points. Adhesion is the first stage and occurs in the first 1-2 hours after seeding followed by spreading, which can develop up to 24 hours after seeding but is more usually established in the first 4 hours. Focal contacts and adhesions also develop up to 24 hours after seeding[220]. The degree of cell survival (viability) is an ongoing process, non-adhered cells are likely to die within the first hour however adherent cells may not have access to the requisite signals for survival and may die at later time points. Proliferation is also an ongoing process but no reports of proliferation up to 4 hours have been made and proliferation up to 12 hours is low. Metabolic activity of cells is a continual process as is the secretion of cellular products (DNA, RNA, proteins, enzymes) and these may be up or down-regulated dependant on the local cellular conditions and signals.

3.3.1.1 Adhesion and Spreading

Studies on the adhesion kinetics of cells plated onto RGD-containing materials have reported various rates of attachment with some studies finding maximal cell adhesion (cell number) within 60 minutes[61,197] and others reporting increasing cell numbers up to 2 hrs [42,61,68,167] or even 5 hours[54,179]. Many of these studies assume that cell adhesion occurs within 60 minutes and the assessment terminates at this time point although a maximum in cell number has not been observed however this is partly due to trying to assess cell adhesion without

contamination from proliferation[189]. Drumheller et al reported increasing cell numbers up 10 hours for high RGD concentrations and up to 50 hours for lower RGD concentrations although it seems likely that some proliferation will have occurred during this time[40].

Average cell area has been shown to increase with time up to the maximum time tested (4 hours)[221] and the percentage of cells that are spread can increase up to 80 hours after seeding[41].

In general the measured adhesion and spreading events increase with time as does the magnitude of the differences between peptide containing materials and negative controls. In some cases at short time points there is no significant improvement gained from the inclusion of an RGD peptide but this may develop at a later time point. For example the adhesion of fibroblasts onto plasma-deposited acrylic acid was greater than the adhesion to the RGD-modified surfaces after 3 hours but after 24 hours the peptide surfaces were significantly better than the control surfaces[67]. Differences between various peptides may also be more clearly observed at longer time points. Osteoprogenitor cells attached to titanium surfaces modified with RGDC and cylco-DfKRG in equal amounts after 1 or 3 hours culture time but after 24 hours the cell numbers were clearly higher on the substrates containing the cyclic peptide than the linear peptide[201]. It is not always the case that spreading increases with time as Yang et al found that some concentrations of peptide coating produced greater cell spreading at 5 hours than at 24 hours[56].

3.3.1.2 Focal Contact Formation

Streeter et al showed that rates of cell attachment to fibronectin and BSA-RGD coatings were similar (see earlier) but that the rates of cell spreading were lower on the RGD coatings. In addition the actin organization on the RGD surfaces was much less pronounced than on the fibronectin coatings and displayed spot-like adhesion structures instead of typical focal adhesions. Singer et al also examined the formation of focal contacts on fibronectin or BSA-RGD coating and found that normal rat kidney fibroblasts formed focal contacts between 1 and 4 hours after seeding[196,207]. The focal contacts on the peptide coated surfaces were unusually associated with fibronectin fibres as well as the usual microfilament bundles that were seen on fibronectin coated surfaces.

3.3.1.3 Viability and Proliferation

It is evident that proliferation has an effect on cell number present at time points less than 24 hours as shown by Varani et al who incubated cells on coatings of poly-L-lysine and PronectinF for 15 minutes and then removed any non-adherent cells prior to continuing cell culture for a further 18 hours[91]. After 18 hours the numbers of cells present on the materials was higher in most cases than after 15 minutes, showing cell proliferation. This means that any studies where the initial or only time point where the cell behaviour is examined is over approximately 12 hours will be examining a mixture of adhesion, viability and proliferation[193].

A rise in cell number over time is due to proliferation and the proliferative index (initial cell number divided by cell number at time t) is often used to describe the cell proliferation. A proliferative index of less than 1 indicates that cell death occurs more frequently than cell division. A proliferative index of 1 probably indicates that cell death occurs at the same rate as cell division rather than implying that no cell division occurs.

There are numerous studies where the proliferation of cells on RGD-materials has been investigated and in many cases there appears to be a definite time point at which proliferation starts. The onset time of proliferation is usually 2-3 days after the seeding of cells[63,189,204] but can be later[49] and the cell number remains fairly constant until this point. Proliferation may not be enhanced by the incorporation of RGD containing peptides even though the initial cell adhesion was promoted[126].

There appears to be some evidence that some RGD-containing materials that can promote cell adhesion are unable to sustain long-term viability of cells. A number of investigations have found that the RGD-containing materials were significantly better than the controls at short time periods but over longer time periods the controls were as good as the peptide-modified materials and there was no statistically significant improvement in cell behaviour[23,26]. Other investigations have found that the viability of cells on RGD-containing materials declines with time such that lower numbers of live cells are found as the time of the experiment increases and that the cell death is greater than cell proliferation)[31,46,59,73,112]. In some cases an initial proliferative spurt can be observed before cell number starts to decline[46,162].

Hsu et al modified PLLA/PLGA scaffolds with collagen and then coated the scaffolds with a RGD-containing fusion protein[24]. At a 12 hour time point there were more chondrocyte cells on the RGD containing materials however after 24 hours their number had declined. The culture on the scaffolds was continued for 6 weeks and the presence of the RGD coating inhibited the cell proliferation and extracellular matrix production. It may be that the fusion protein containing RGD de-adsorbed from the surface and reduced the cell number present over that of the controls by providing soluble competition for integrin receptors.

It has been found that the number of cells present on SAMs containing RGD declined after 12 hours such that, after 24 hours the number of cells was equivalent to that on the scrambled peptide control, even though there had been an initial increase in cell number (1, 4 and 12 hour time points)[170]. Cells cultured on fibronectin coatings under the same conditions maintained their viability. The loss of cell adhesion was attributed to the failure of the cells to secrete fibronectin. However this implies that the adhesion, after a 12 hour time point, is due to adhesion onto synthesised proteins, which seems unlikely. Kao et al found that the cell number dropped between 1 and 24 hours of culture, stabilised and then proliferation occurred after 180 hours in culture[101]. Park et al found that although the viability of hepatocytes encapsulated in RGD-containing hydrogels declined with time the rate of loss of viability was less than that observed with the non-modified hydrogels[212].

Studies that investigated both the proliferation by counting cell numbers and the viability via tryphan blue showed that the total cell number in RGD-containing hydrogels increased over time but that the relative viability of the cells decreased[75]. Another study that investigated the viability of cells over time showed that the viability of insulinoma cells encapsulated in poly(Nipaam-co-PEG) hydrogels declined steadily up to 7 days at which point the levels of viability remained constant[33]. This implies that there is a certain population of the cells that have not successfully grafted into the material and it is a matter of time before they die whilst the remaining population is successful. A similar result was also found by Phillips et al who investigated the monocyte cell numbers on RGD-modified PEG hydrogels and found the decline in cell numbers

was similar to that of tissue culture plastic and reached a plateau after approximately 100 hours[112].

It should be noted that some peptide sequences are more likely to promote proliferation than others as shown by Verrier et al who compared the number of osteoprogenitor cells over time on cyclo-DFKRG modified surfaces and GRGDSPC modified surfaces[92]. After 24 hours the numbers of cells on each material were similar showing similar promotion of attachment by both peptides but after 48 hours the numbers of cells on the cyclic peptide modified surfaces were significantly greater than on the linear peptide modified surfaces.

3.3.1.4 Differentiation

Hepatocytes were grown on polystyrene coated with different peptides (P-2, PronectinF and fibronectin) and it was shown that all peptides promoted similar levels of cell attachment and that some promoted high levels of DNA synthesis but low levels of albumin secretion (differentiation marker)[93]. Conversely those that were not as successful at promoting DNA secretion maintained a higher degree of differentiated function showing an inverse relationship between growth and differentiation. Other studies have found that the incorporation of RGD in Nipaam-co-PEG hydrogels leads to enhanced hepatocyte albumin and urea synthesis[32,212]. It has also been shown that insulin secretion of insulinoma cells is also upregulated by the presence of RGD in poly(Nipaam-co-PEG) hydrogels[33].

3.3.2 Experimental Design

The experimental parameters involved in the assessment of RGD-materials to determine whether they are fit for purpose are self-evidently determined by the purpose for which the materials are designed. As a consequence of the wide range of purposes there are a wide range of experimental parameters that are utilised in the analysis of the biomaterials. However alterations in these experimental parameters can produce large effects in whether a material is deemed successful and some of the decisions made on how to conduct a biological evaluation of RGD-containing materials may be significant.

3.3.2.1 Cells

A large number of different cell types and sources have been utilised to assess RGD-containing biomaterials. These include primary cells derived from animal or human sources and immortalised cell lines. The responses of primary cells are more likely to be similar to cells in their natural *in vivo* environment. However generating reproducible data from these cells can be more difficult than with cell lines as the source of the cells, passage number and culture conditions alter the cellular responses. Examples of cell types that have been investigated include fibroblasts, osteoblasts, epithelial cells, endothelial cells, keratinocytes, chondrocytes, hepatocytes, neurites, macrophages, melanoma, mesenchymal stem cells and osteoprogenitor cells. It should be noted that, due to the wide range of cells expressing RGD-binding integrins, the incorporation of an RGD peptide into a non-fouling material will also mediate the adhesion of immune/ inflammatory cells such as macrophages[112].

Cell Type

Different cell types express different integrin expression profiles and consequently the behaviour of separate cell types to the same RGD-containing material may be changed. Studies that have investigated the behaviour of various cell types have shown differences between the cell adhesion in terms of cell number and spreading and also in the magnitude of improvement caused by the incorporation of the peptide over the negative control[81].

This is illustrated by the attachment of various cells to RGD-modified and non-modified collagen/GAG matrixes[222]. Fibroblasts attached well to the non-modified surface whereas keratinocytes and endothelial cells did not. The immobilisation of RGD on the matrixes caused a significant improvement in the numbers of keratinocytes and endothelial cells adhered but the extent of the improvement was much smaller with fibroblasts. In addition, despite the same numbers of each type of cell being seeded, the amount of adhered fibroblasts is twice that of the other cell types on all materials.

Kurihara et al investigated the adhesion of fibroblast, cancer and neuronal cells to coatings of multiple RGD repeat sequences (2, 21, or 43 repeats) or fibronectin. Only the neuronal cells adhered better to $(RGD)_{43}$ coatings than fibronectin coatings and they also displayed better responses to lowering the

coating concentration than the other cell types. Fibroblast cells showed improved cell adhesion to all peptide coatings whereas the cancer cells only responded to the longer repeat sequences and the neuronal cells only responded to the $(RGD)_{43}$ coating.

Cell Source

Even within a cell type the source of the cells may affect the reported results although the responses of the cells cultured on the material usually follow the same trends the magnitude of the response can be altered. This was shown by Singer et al who cultured various fibroblast cell lines on surfaces coated with BSA that was subsequently modified with RGD-containing peptide[207]. The formation of focal contacts of various fibroblast cell types on BSA-RGD and fibronectin coatings was investigated and it was found that Balb/c 3T3 (mouse clone A31) cells were incapable of forming focal contacts on the peptide coated surfaces. NRK (normal rat kidney) and Nil 8 (normal hamster) fibroblasts formed numerous focal contacts on the same surfaces and produced significantly more focal contacts on fibronectin coated surfaces than the Balb/c 3T3 cells.

In cases of primary cells the donor source may affect the reported results as Marletta et al showed when they investigated the cell adhesion of primary osteoblasts derived from patients of different ages. Cells derived from younger patients tended to cover a larger percentage of the area of poly(caprolactone) coated with RGD and also tended to show a larger increase on the RGD coated materials over the non-coated materials[57]. In addition the length of time that primary cells are cultured for affects their integrin expression and hence their affinity for RGD peptides[223]. The trends in integrin expression as passage number increases for human chondrocytes are shown in Figure 3-13 below.

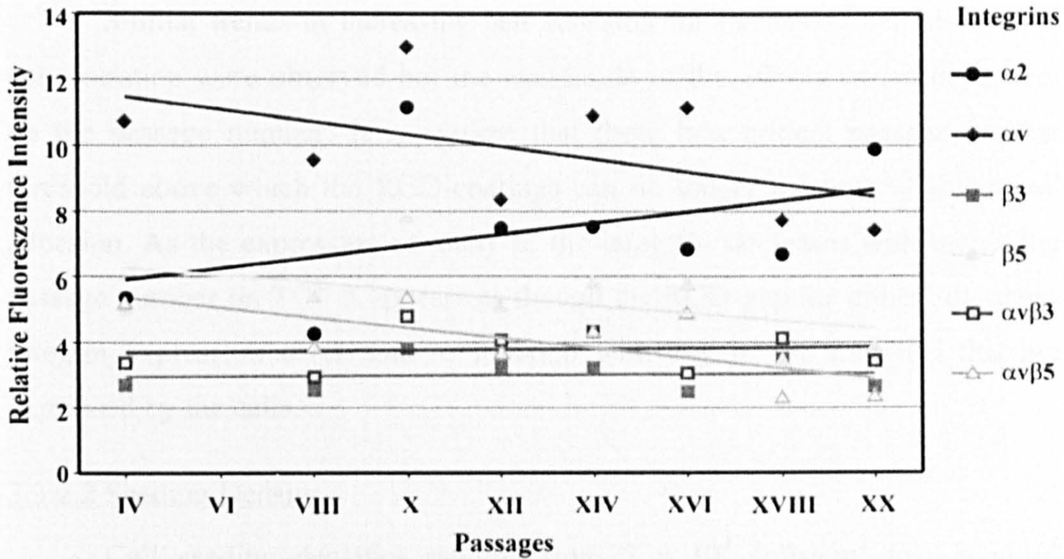


Figure 3-13 Trends of integrin expression during monolayer cultivation of human articular chondrocytes. Reproduced from Jeschke et al[†,223].

From Figure 3-13 it can be seen that the α_v integrin expression decreases as the passage number increases. Jeschke et al also investigated the effect of increasing passage on cell adhesion to RGD-peptide coated surfaces. The seeding efficiency increased with increasing passage number from passage 10 to passage 18 but then fell dramatically for passage number 20 as shown in Figure 3-14.

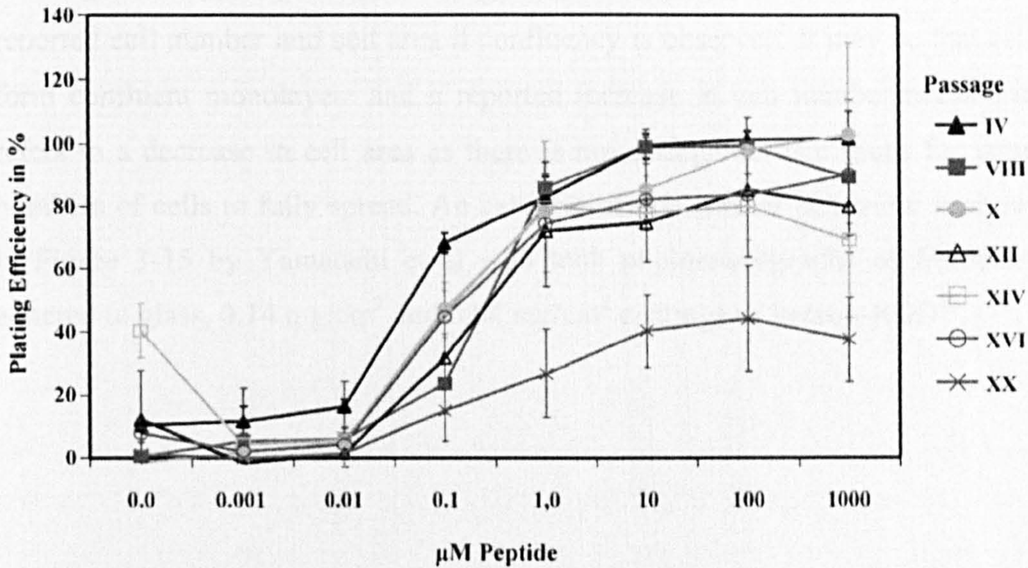


Figure 3-14 Adhesion of human articular chondrocytes on RGD-peptide coated surface in the course of monolayer cultivation. Reproduced from Jeschke et al[‡,223].

---

†,‡ Reproductions of Figure 3(†) and Figure 6 (‡) from "Jeschke, B., Meyer, J., Jonczyk, A., Kessler, H., Adamietz, P., Meenen, N. M., Kantlehner, M., Goepfert, C. & Nies, B. RGD-peptides for tissue engineering of articular cartilage. *Biomaterials* **23**, 3455-3463 (2002)" with permission from Elsevier Ltd.

Similar trends in increasing cell adhesion for increasing peptide-coating concentration were observed but the magnitude of the effects varied dependent on the passage number. It is evident that there is a critical passage number threshold above which the RGD-coatings can no longer promote effective cell adhesion. As the expression of many of the integrins decreases with increasing passage number on TCP it appears as though the RGD-peptide either stimulates integrin expression or is able to function with any of the integrins that are expressed by the cells.

3.3.2.2 Seeding Density

Cell seeding densities ranging from 5 x 10^3 cells/cm^2 to 1.8 x 10^6 cells/cm^2 have been employed to test the effectiveness of RGD-containing biomaterials. There is some evidence showing that the seeding density may have an effect on the reported outcome. High cell seeding densities may produce a loss of viability due to confluency promoting contact-mediated cell death[73].

High cell seeding densities have been shown to reduce the secretion of endothelin-1 on PronectinF coatings due to contact inhibition as the cells form a monolayer[214]. In addition high cell seeding density may have an effect on the reported cell number and cell area if confluency is observed. It may be that cells form confluent monolayers and a reported increase in cell number necessarily refers to a decrease in cell area as there is not enough surface space for larger numbers of cells to fully spread. An example of this type of behaviour is shown in Figure 3-15 by Yamauchi et al who took photomicrographs of fibroblasts adhered to glass, 0.14 mg/cm^2 and 0.64 mg/cm^2 coatings of keratin-RGD[52].

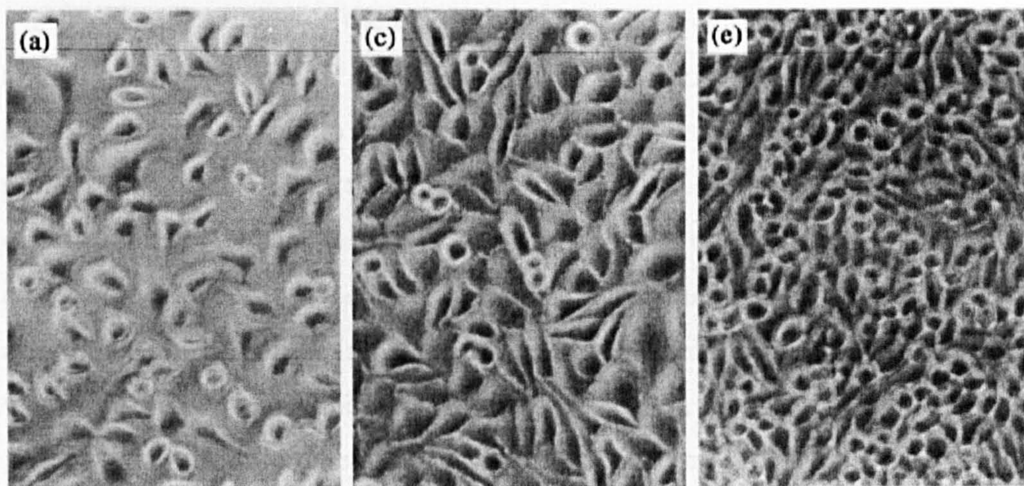


Figure 3-15 Photomicrographs of fibroblasts adhered to glass (a), 0.14mg/cm^2 (c) and 0.64cm^2 coatings of keratin-RGD (e) after 24 hours. Note that the seeding density was 2x10^6 cells per dish of diameter 3.5cm (equivalent to ~2.08x10^5 cells/cm^2). Reproduced from Yamauchi et al[†,52].

3.3.2.3 Culture Conditions

The manner in which cells attach to surfaces can be altered by the conditions under which they are cultured. One of the most common variables that can be altered is the presence or absence of serum in the media. There is a growing awareness of the need to minimise the use of animal-derived products in the production of biomaterials seeding with cells and consequently the behaviour of cells in serum-free media is often investigated.

Serum-Containing vs. Serum Free Media

The presence of serum in the media can alter the cell adhesion profile over time as shown by Lin et al[61]. In serum-containing media maximal cell adhesion is seen after 40 minutes in comparison to the same materials in serum-free media where the number of attached cells rises steadily over the time period assessed (up to 240 minutes). Consequently in serum-containing media the effects of RGD can be seen more clearly at short time points than in serum-free media where longer times will emphasise the differences between substrates.

† Reproduction of Figure 4 from "Yamauchi, K., Hojo, H., Yamamoto, Y. & Tanabe, T. Enhanced cell adhesion on RGDS-carrying keratin film. *Materials Science & Engineering C* **23**, 467-472 (2003)" with permission from Elsevier Ltd.

The magnitude of the effect caused by the inclusion of a peptide may be altered by the presence or absence of serum. The presence of serum can increase the effect observed due to increased cell viability[73] or decrease the effect due to protein effects obscuring the effects of the peptide. In some cases the positive effects of the inclusion of an RGD sequence can only be seen in serum-free media. Gümüşderelioğlu et al observed a significant improvement in the cell attachment to non-woven polyester fabric when it was modified with RGD only when the cells were adhered in serum-free media and not when serum was present[54]. In addition the dependence of the cell adhesion on the RGD concentration is more clearly shown in serum-free media as shown by Brandley et al[69]. The cell attachment appeared to show no dependence on RGD concentration in serum-containing media but a clear dependence was shown in serum-free media. In addition low concentrations of peptide appear to be less effective in serum-free media than in serum-containing media[121].

In addition serum may alter the expression profile of extracellular matrix components, proteins or other cell secretion products. The presence of serum appears to inhibit the production of proteins as shown by Malek et al who investigated the production of endothelin-1 by endothelial cells on PronectinF coatings when cultured with serum concentrations ranging from 0 – 10%[214].

Dynamic Cell Culture

As well as the increased attachment of cells in terms of cell number and the increased spreading the incorporation of an RGD peptide can improve the adhesion strength. Cells are more likely to be retained in dynamic environments where stresses are placed on the cells such as fluid pressure in replacement of blood vessels[65,188,205,224,225] or mechanical flexing[120,121]. The presence of RGD peptides has been shown to increase the force necessary to detach cells from a substrate[123,175,176].

3.3.3 Analysis Methods

There are two main types of analysis methods for determining the ability of biomaterials to promote various aspects of cellular behaviour, visual methods and assay-based methods. Assay based methods are quantitative and show the relative activities of cells whereas visual methods can be used in a qualitative or quantitative manner and show the relative morphologies or numbers of cells.

3.3.3.1 Visual Methods of Analysis

Image Generation

Images of cells can be generated by phase contrast microscopy, immuno-fluorescent microscopy, confocal microscopy and scanning electron microscopy (SEM). Phase contrast and immuno-fluorescent microscopy require the substrate on which the cells are adhered to be optically transparent.

Image Analysis

When images of cells grown on substrates have been captured the images are often analysed to try to provide a quantitative measure of the success of the material. The information that can be gained from images of cells includes cell number, cell area, cell shape (including filopodia formation and neurite extension) and the area of material covered by cells. From this information the data can be collected and summed up in a variety of ways.

The number of cells per unit area and the percentage adhesion (cells per unit area divided by applied number of cells per unit area) are in common use as measures of cell behaviour on novel biomaterials. However this information does not give an indication of the viability of the cells and does not differentiate between healthy cells and rounded cells that are likely to die soon.

The morphology of the cells on the substrates gives a better indication as to the viability and normal behaviour of the cells however it is more difficult to represent this as numerical data. Percentile area charts have been used to indicate the numbers of cells that fall into various categories of cell area[96] however this method does not indicate the numbers or densities of cells adhering to a substrate.

In references where data is presented containing only one aspect of the image such as cell number or the cell shape the results should be treated with caution. The general trends shown by the image analysis will probably be accurate however the differences between substances may be magnified or reduced dependent on the analysis as shown by Massia et al in Figure 3-16 below. In this case endothelial cells appear to respond poorly to the linear peptide as the cell numbers are ~50% of the control but when the cell area is analysed the linear peptide appears to promote good cell spreading.

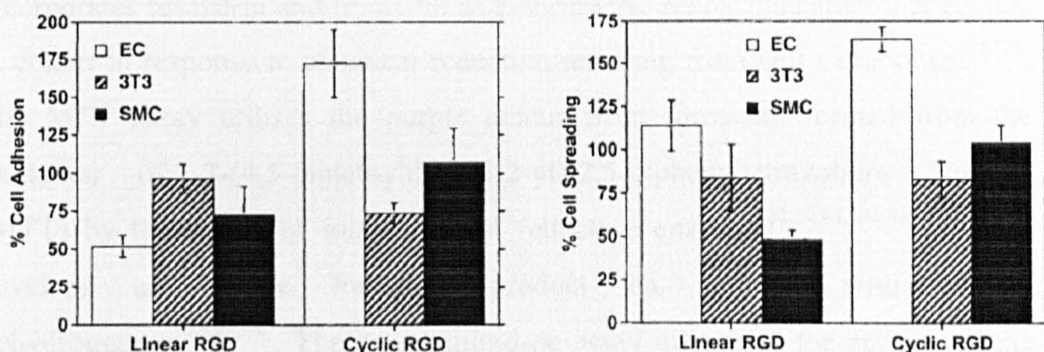


Figure 3-16 Comparison of EC, 3T3 and SMC adhesion on substrates containing surface grafted linear and cyclic RGD peptides. Left - % cell adhesion. right - % cell spreading. Reproduced from Massia et al [†,187]

In addition artefacts can be produced from the experimental conditions utilised such as the seeding density. If cells are confluent or over-confluent the mean cell area will be reduced as cells will not have enough space in which to fully spread (see section 2.2.2).

3.3.3.2 Assay Based Methods of Analysis

Assay based methods provide a quantitative measure of the amount of cellular components, products or the activity of cellular process. The cellular products generated can be found in the cell culture media or deposited on the material's surface. The amount of cellular products deposited on a surface will be dependent on the characteristics of the substrate as well as the rate of production. The rate of production can be assessed by analysing the amount of cellular products generated at various time points.

---

† Reproductions of Figure 6 and Figure 7 from "Massia, S. P. & Stark, J. Immobilized RGD Peptides on Surface-Grafted Dextran promote Biospecific Cell Attachment, *Journal of Biomedical Materials Research* **56**, 390-399 (2001)" with the permission of John Wiley & Sons Ltd.

Assays for cellular components cannot distinguish between live cells and dead cell material deposited on a surface. Examples of cellular components that have been utilised to assess the amounts of cells on RGD-containing biomaterials include total DNA[25,30,167], nucleic acid assay (CyQuant)[59,78,93,170], total RNA[34] and total protein assay[120,226].

Assays that depend on the current activity of cellular processes include Alamar blue, CCK-8, hexosaminidase and MTT assays. The Alamar blue assay incorporates resazurin and resarufin as colorimetric redox indicators that change in colour in response to chemical reduction resulting from cell metabolism[57,224]. The MTT assay utilises the purple colour from formazan formed from the oxidation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the action of mitochondrial reductase enzymes[31,50,87,181,227]. CCK-8 develops an orange formazan product on reaction with cellular dehydrogenases[26,89,162]. The hexosaminidase assay quantifies the activity of the lysosomial enzyme N-acetyl-β-D-hexosaminidase using p-nitrophenyl N-acetyl-β-D-glucosaminide[43,44,90,92,228]. Other assays for cellular processes include lactate dehydrogenase activity[68-70,212] and chromogenic substrate solution[201].

Some assays can show the levels of cell differentiation as the cellular process is only present in certain cell types and the activity of the process or the amount of products can be quantified. Urea synthesis in hepatocytes can be quantified by the Berthelot method[88,229,230] and alkaline phosphatase production in osteoblasts can be assessed with p-nitrophenyl phosphate[25,34,73]. Dopamine secretion in pheochromocytoma cells was measured by HPLC with an electrochemical detector using 5% buffered methanol[178]. Hydroxyproline content is a marker of collagen synthesis and can be determined by oxidation with chloramine and development with p-dimethylaminobenzaldehyde[30,231].

Enzyme-linked immunosorbent assay (ELISA) can be used to determine amounts of cell products for which there is an antibody. A peroxidase-conjugated antibody is bound to the cell product and the amount of peroxidase is quantified with a calorimetric assay. Albumin synthesis[93,212], endothelin-1 production[214] and osteocalcin[34] have been quantified on RGD-containing substrates using ELISA.

3.3.3.3 Comparison of Methods

In general the trends observed by varying the method of analysis are similar however the magnitude of the effects observed may be different. This was shown by Smith et al where the trends in increasing peptide concentration were similar however by MTT assay the highest concentration of RGD gives a result comparable to TCP whereas by cell count the material is clearly poorer than TCP as shown in Figure 3-17 below[73].

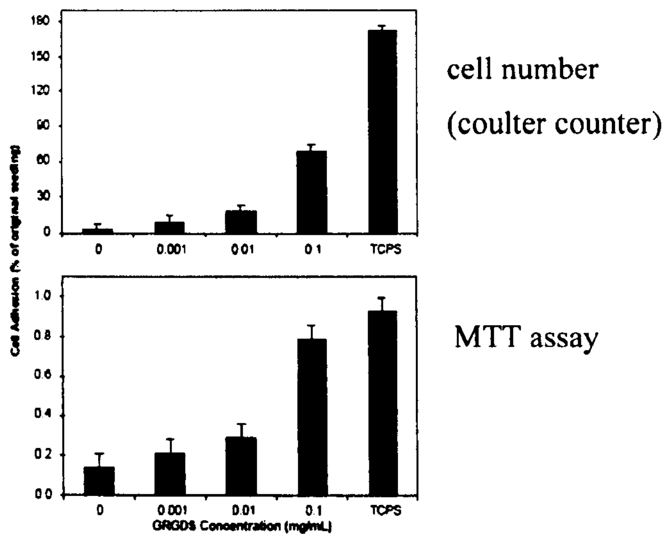
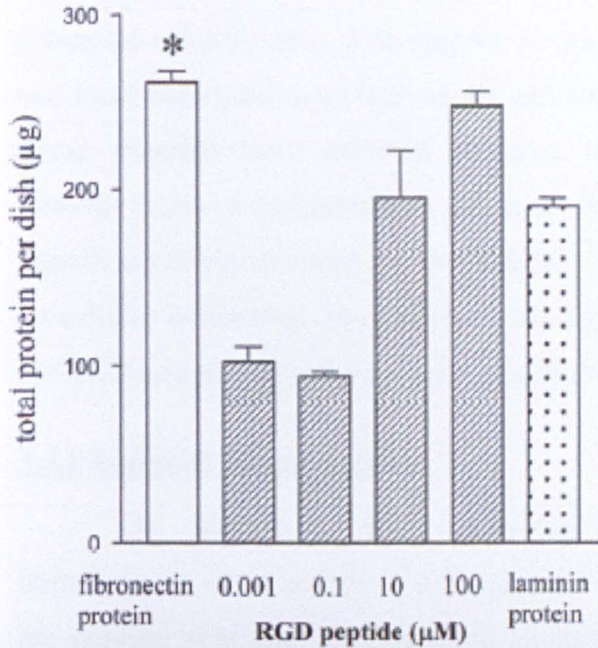


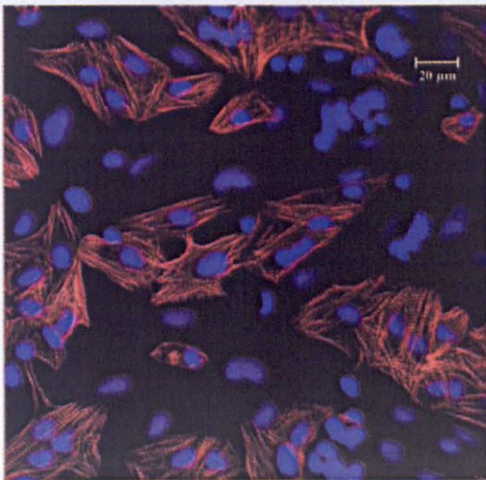
Figure 3-17 24 hour adhesion of C2C12 cells to RGD-modified Nipaam-co-NASI films. Reproduced from Smith et al[†,73]

Similar observations where assay based methods and visual based methods have been compared show that this is a fairly common phenomenon[120,227,232]. In particular the MTT assay gives an elevated response for some materials compared to the cell number probably due to an increased respiration rate for loosely adhered cells.

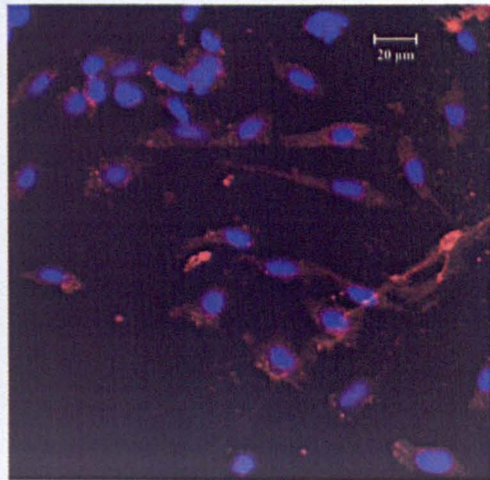
† Reproduction of Figure 3 from "Smith, E., Yang, J., McGann, L., Sebald, W. & Uludag, H. RGD-grafted thermoreversible polymers to facilitate attachment of BMP-2 responsive C2C12 cells. *Biomaterials* **26**, 7329-7338 (2005)" with the permission of Elsevier Ltd.

A total protein assay conducted on myocytes seeded onto RGD-modified silicone membranes or fibronectin coated surfaces showed equivalent cell numbers on both surfaces. However immuno-fluorescent staining with phalloidin for actin showed that the fibronectin surface clearly promoted actin filament organisation but the RGD-modified surfaces did not as shown in Figure 3-18.





Myocytes on fibronectin



Myocytes on RGD peptide

Figure 3-18 Adhesion of cardiac neonatal myocytes to peptide-modified silicone and fibronective coated silicone (48 hours) assessed by total protein assay (above) and phalloidin staining (below). Reproduced from Boateng et al[†,120].

† Reproduction of Figure 2 from "Boateng, S. Y., Lateef, S. S., Mosley, W., Hartman, T. J., Hanley, L. & Russell, B. RGD and YIGSR synthetic peptides facilitate cellular adhesion identical to that of laminin and fibronectin but alter the physiology of neonatal cardiac myocytes. *American Journal of Physiology - Cell Physiology* **288**, 30-38 (2005)" used with permission from APS.

3.4 Effects of RGD Design on Cell Behaviour

As shown in section 3.2 there are numerous strategies for immobilising RGD peptide sequences on a wide assortment of materials with different intrinsic abilities to promote various aspects of cell behaviour. In addition there is considerable variety in the peptide sequence containing RGD that can be presented towards cells with respect to the choice of peptide length, sequence and local organisation as well as the concentration and immobilisation strategy. These peptides have different affinities for integrins as determined by the flanking residues, conformation and accessibility towards integrins. More affine ligands are likely to require a lower density of peptide to achieve the same effect on cellular behaviour. The effects of the design parameters for the synthesis of the RGD-containing materials are discussed here.

3.4.1 Material Design Effects

The analysis of RGD-containing biomaterials usually refers to the improvement of an aspect of cellular behaviour obtained from the inclusion of the peptide over that of a negative control. The negative control is usually a substrate that is similar to the peptide-modified substrate but lacking the active ligand. The controls can include a scrambled or substituted peptide or can be devoid of any peptides. The improvement that can be obtained from the inclusion of the peptide can be dependent on the background substrate to which the peptide is added. In addition the presence of the peptide can alter the physical and chemical properties of a material and affect the biological properties of the material through non-specific means. This section deals with the effects that the background materials and the synthesis methods have on the ability of RGD to promote cell attachment.

3.4.1.1 Proof of RGD Specificity

The inclusion of RGD in a biomaterial may have effects on the biological properties by means other than the binding of the peptide to integrins. The peptide may change the chemical and physical properties of the material (surface roughness, phase separation, water content, electrostatic properties etc) and through them the biological properties by a modified protein adsorption profile. Consequently the assessment of the biomaterials often includes a methodology to

determine whether the effects of the peptide are due to specific integrin binding or more generally through other means.

Peptide-Containing Controls

One method of determining whether the presence of the peptide has altered the properties of the biomaterial is to compare the RGD-containing material to one containing a peptide sequence with a substituted peptide or a scrambled peptide. A substituted peptide is one in which one of the amino acids has been replaced with another amino acid with similar properties such as the replacement of D (aspartic acid) with E (glutamic acid). A scrambled peptide is one in which the amino acids remain the same but the order of the active sequence is scrambled. This method has the advantage of categorically confirming whether any increase in cell adhesion is due to integrin binding but the disadvantage of requiring synthesis of additional peptide and materials. According to Olbrich et al there does not appear to be any differences in cell behaviour observed between the substituted peptide RGE and the scrambled peptide RDG[128].

In the majority of cases where a substituted or scrambled peptide has been investigated the cell behaviour is not improved over the non-peptide containing control however there are some exceptions. Park et al incorporated GCGYGRGDSPG or GCGYGRDGSPG into halouronic acid and PEG based hydrogels in amounts approximating to 60μmol/g. Cell activity was promoted on the networks derivatised with the scrambled peptide although not to the same extent as on the networks derivatised with the active peptide.

Soluble Peptides

Another method to determine the specificity of cell adhesion is to utilise a soluble peptide that blocks the integrin binding sites and hence prevents cell adhesion by RGD-integrin specific routes. The soluble peptide may be added to the cells prior to seeding, to the media as the cells are seeded, or to the media after a set time period has elapsed.

It should be noted that a high concentration of soluble peptide may be required for competitive binding as shown by Porte-Durrieu et al, who incubated endothelial cells with soluble RGDC for 30 minutes immediately prior to seeding

the cells on silica grafted with RGDC. It was found that 5μg/ml of the soluble peptide was insufficient to compete with the immobilized peptide however 1000μg/ml of the soluble peptide caused a significant decrease in the cell adhesion. The inhibition of cell adhesion appears to be proportional to the amount of soluble RGD utilised[99]. This was confirmed by Hayman et al who also showed that different cell types can respond differently to equivalent concentrations of soluble RGD peptide[161].

The remodelling of surfaces by cells was illustrated effectively by Ebara et al who used soluble RGD to detach cells from surfaces they had been cultured upon for varying lengths of time. After 4 and 24 hours of culture the cells detached more rapidly and to a greater extent than after longer periods of culture[76].

Integrin binding sites can also be blocked using antisera that can be chosen to only bind to certain integrins[53,129,169]. This shows the integrin specificity as well as the selectivity of the RGD-integrin binding effects.

1.1.1.1 Substrate Effects

Alterations in the ability of the control substrate to promote that aspect of cellular behaviour may modify the level of improvement gained from the RGD peptide. For example control substrates may be able to promote high levels of cell attached through protein adsorption but the proteins may hinder the integrin binding to peptides and decrease their effectiveness. The ability of the control substrate and the effect of the peptide on an aspect of cell behaviour could be competitive, cumulative or cooperative.

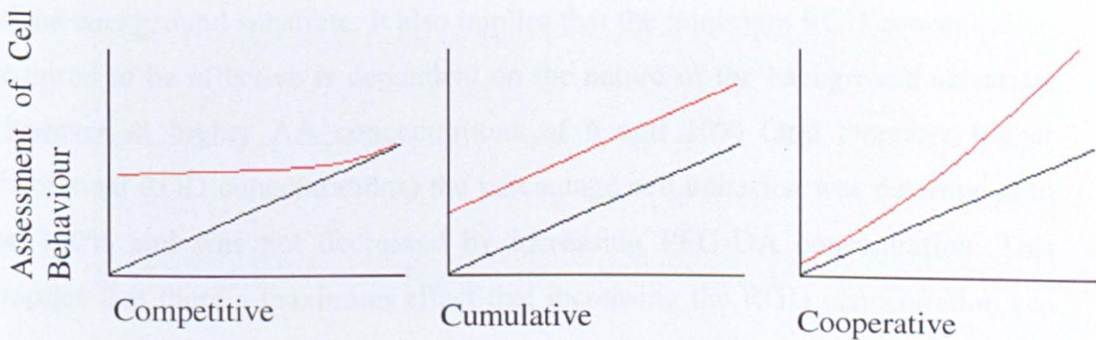


Figure 3-19 Effect of ability of control substrate and RGD-peptide inclusion on the cell behaviour. Black lines – ability of control to promote an aspect of cell behaviour, red lines – improvement in cell behaviour caused by the inclusion of an RGD-peptide.

The ability of the RGD-peptide to promote an aspect of cell behaviour is considered in terms of the improvement over a control substrate and the overall level of the cell behaviour. Significant improvements in an aspect of cell behaviour from the inclusion of an RGD-peptide can still result in materials that behave poorly in comparison to other materials if the control substrate gave poor levels of cell behaviour. There is only a limited amount of literature wherein the effect of changing the background substrate on the effect caused by RGD inclusion has been investigated.

Chemical Effects

It is likely that the chemical composition of a substrate will affect the ability of an immobilised peptide to promote cell adhesion and other aspects of cell behaviour as the environment that the peptide is in will affect the peptide conformation as well as the approach of cells and integrins. In addition the ability of the peptide to perform may be affected by the ability of the bulk substrate to adhere proteins.

Drumheller et al synthesised semi-interpenetrating networks from PEG-DA, acrylic acid and trimethylolpropane triacrylate and derivatised the surfaces with GRGDS in concentrations that could be controlled by altering the % of acrylic acid incorporated[40]. At 1% AA (and theoretical 1% RGD modification) the percentage cell adhesion was ~83% when 0.1 g/g PEG-DA was utilised but the percentage cell adhesion decreased to 75% and 64% when the PEG-DA concentration was increased to 0.2 and 0.4 g/g respectively. This shows that the effectiveness of a given concentration of peptide can be modified by the nature of the background substrate. It also implies that the minimum RGD concentration required to be effective is dependent on the nature of the background substrate. However at higher AA concentrations of 6 and 10% (and therefore higher theoretical RGD concentrations) the percentage cell adhesion was determined to be 100% and was not decreased by increasing PEG-DA concentration. This implies that there a maximum effect that increasing the RGD concentration can have on the cell adhesion and further increases in peptide concentration will not improve the cell behaviour but may protect the ability of the peptide to work in adverse conditions. The concentrations of peptide in the materials were assessed to be 12, 66 and 110pmol/cm^2 by radiolabelling. However these figures denote

the amount of peptide immobilised per square centimetre of network. It seems likely that the surface accessible concentration of RGD is significantly lower as this amount of peptide will be spread throughout the bulk of the porous network. The thickness of the networks was not stated so it is not possible to determine the surface concentration. There are other severe limitations of this work as well; the percentage cell adhesion was determined to be 100% after 24 hours culture. This figure seems unrealistic as it seems unlikely that 100% of cells would have adhered and during a 24 hour period some proliferation will have occurred such that the percentage adhesion should be slightly higher or lower than the 100% point. In addition as the amount of PEG-DA was altered the overall mass of the network was also altered such that the amount of RGD was diluted over a larger area. This makes it difficult to tell whether the decrease in cell adhesion is due to a decrease in effective RGD concentration or due to the change in background substrate.

In one system self-assembling monolayers were created where the amount of peptide could be precisely controlled and the micro-environment could be altered by using different lengths of oligo(ethylene glycol) chains on the non-modified species forming the monolayers[171]. It was found that as the chains grew longer the cell number and average cell area decreased linearly. In addition at the shortest PEG chain length the three different concentrations of RGD performed equally well whereas at longer chain lengths greater concentrations of RGD promoted higher levels of cell attachment. This appears to support the work of Drumheller et al in which it was implied that the RGD concentration only affects the cell behaviour below a critical threshold of substrate effects on cell behaviour. It appears that increasing the chain length of ethylene glycol units has hindered the access of peptide sequences towards integrins such that the concentration of accessible peptides is lowered below a critical threshold and becomes crucial to the extent of cell adhesion and spreading. The change in micro-environment has altered the accessibility of the peptides and consequently the peptide concentration required for maximal cell response has increased.

Lee et al produced self-assembling monolayers with various surface chemistries, modified these with RGD and found that the peptide incorporation produced a small improvement in the cell adhesion strength regardless of the background surface as shown in Figure 3-20 below[176]. This implies that the

effect of RGD-inclusion on cell detachment strength is cumulative with the effect of the control substrate.

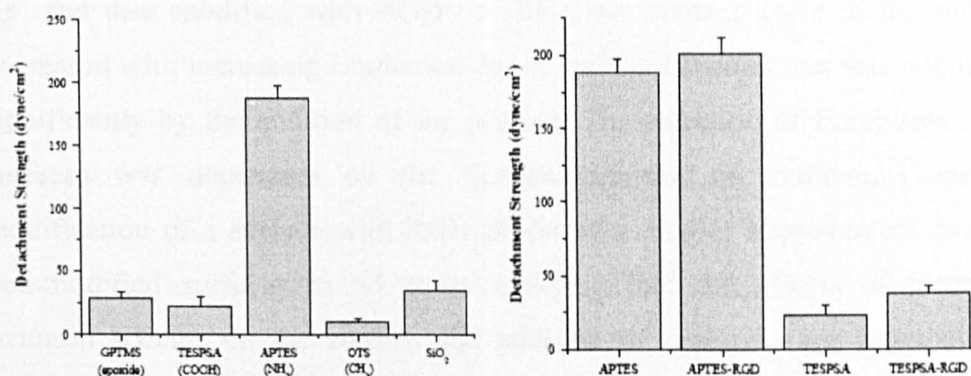


Figure 3-20 Cell adhesion strength to self-assembling monolayers with various chemistries (A) and the self-assembling monolayers with RGD attached (B). Reproduced from Lee et al[†,176].

Hydrogels of PEG and HEMA have been derivatised with RGD or the charged monomer species 2-sulfoethyl methacrylate (negative) or 2-methyacryloxy ethyltrimethyl ammonium chloride (positive) incorporated[81]. The concentration of the charged species was fixed at 200mM and the peptide density was not analysed. The incorporation of both charged species and RGD increased the levels of cell attachment over the neutral control hydrogel. The positively charged species increased the levels of cell attachment more than the peptide modification. It is not known whether an increased concentration of RGD could produce a level of cell adhesion equal to or greater than that produced by the positively charged monomer. It would be interesting to see whether the addition of RGD to charged hydrogels would promote an additional increase in cell adhesion when the two strategies are utilised in combination.

† Reproduction of Figure 3 from "Lee, M. H., Brass, D. A., Morris, R., Composto, R. J. & Ducheyne, P. The effect of non-specific interactions on cellular adhesion using model surfaces. *Biomaterials* **26**, 1721-1730 (2005)" with permission from Elsevier Ltd.

Polyethylene surfaces were oxidised by ion-beam irradiation with Ar^+ or Kr^+ and then modified with RGD[233]. The water contact angle of the surfaces decreased with increasing irradiation due to oxidised species and was not altered significantly by the addition of the peptide. The adhesion of fibroblasts to the surfaces was dependent on the fluence (amount of irradiation) and the modification of a surface with RGD produced a similar improvement over the non-modified surfaces in all cases implying that the effects of increasing oxidised species on the surface and addition of peptide were cumulative as shown in Figure 3-21.

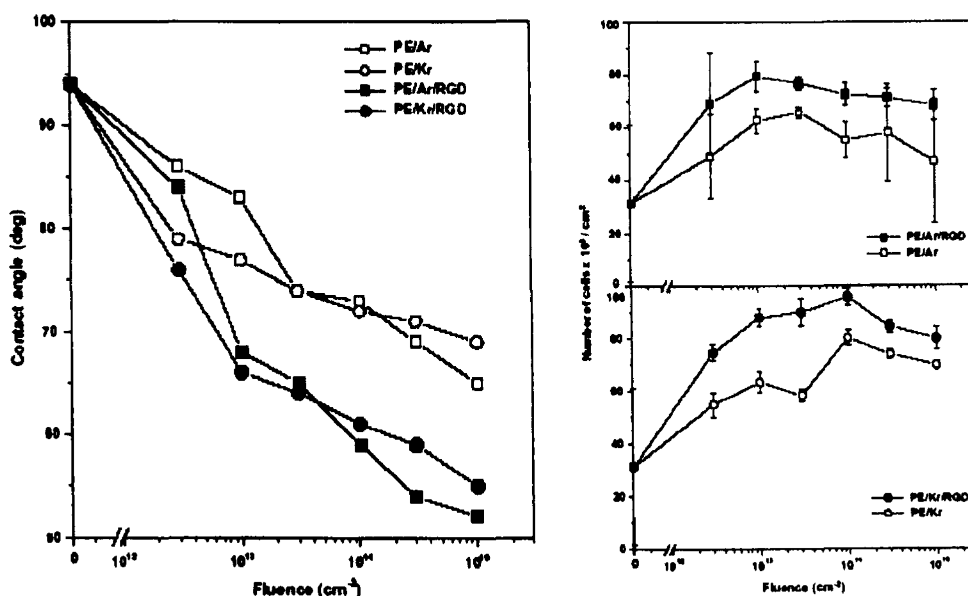


Figure 3-21 Water contact angle (A) and fibroblast adhesion numbers (B) on polyethylene irradiated with Ar^+ or Kr^+. Reproduced from Ročková-Hlaváčková[†,233].

Physical effects

Surface roughness has an effect on the ability of surfaces to adhere cells and also an effect on the ability of RGD to promote the cell adhesion. Schuler et al showed that on poly(lactic acid-graft-polyethylene glycol) substrates smooth surfaces coated with RGD were more effective than rough surfaces coated with RGD[113]. The effect of the surface topography and the addition of the active peptide sequence appeared to be cumulative when compared to the scrambled peptide sequence.

† Reproduction of Figure 2 and Figure 4 from "Rocková-Hlavácková, K., Svorcik, V., Bacakova, L., Dvorankova, B., Heitz, J. & Hnatowicz. Bio-compatibility of ion beam-modified and RGD-grafted polyethylene. *Nuclear Instruments and Methods in Physics Research B* **225**, 275-282 (2004)" with the permission of Elsevier Ltd.

Nano-amorphous calcium phosphate, nano-crystalline hydroxyapatite and conventional hydroxyapatite were all modified with RGD and the adhesion of osteoblasts to the surfaces was examined[200]. As can be seen in Figure 3-22 the modification of the conventional hydroxyapatite with RGD produced an increase in cell adhesion which gave rise to a cell density comparable to that produced by the non-modified nano-amorphous material. The addition of RGD increased the cell adhesion in all cases and it is interesting to note that the largest increase was obtained on the materials that performed best without the modification. This implies that the effects of substrate and the effects of RGD-inclusion were cooperative in determining the numbers of adhered cells per cm^2 in this case.

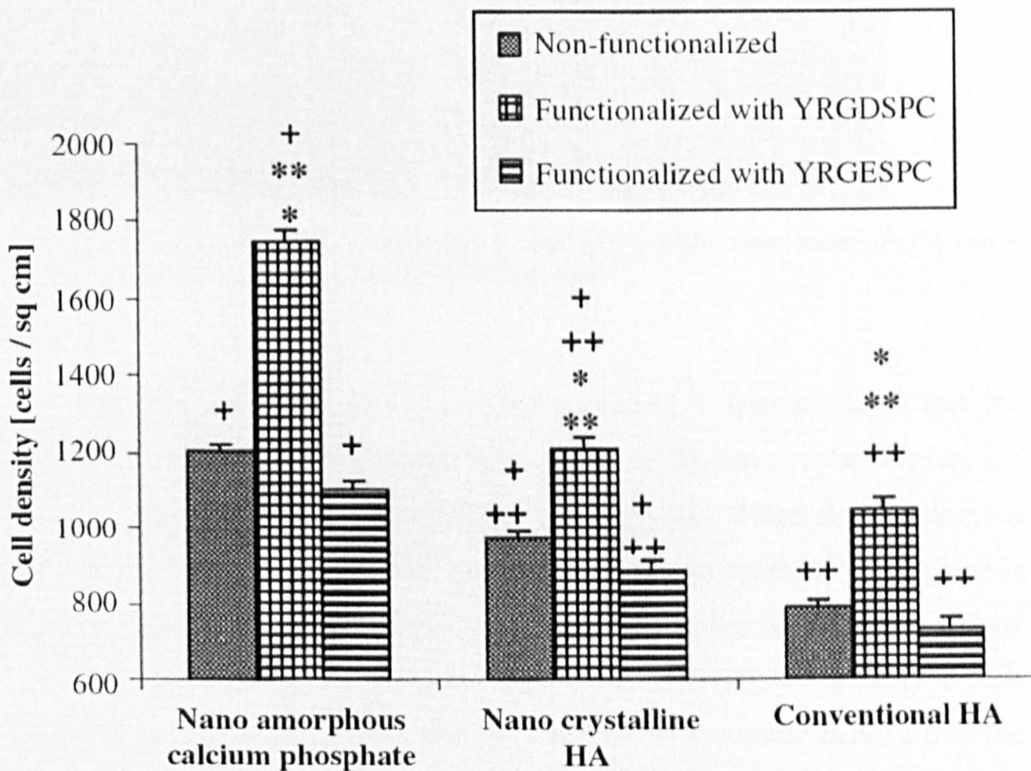


Figure 3-22 Osteoblast adhesion to calcium phosphate and hydroxyapatite materials with differing levels of crystallinity. Reproduced from Balasundaram et al[†,200].

† Reproduction of Figure 5 from "Balasundaram, G., Sato, M. & Webster, T. J. Using hydroxyapatite nanoparticles and decreased crystallinity to promote osteoblast adhesion similar to functionalizing with RGD. *Biomaterials* **27**, 2798-2805 (2006)" with permission from Elsevier Ltd.

The rigidity of a material will also have an effect on some aspects of the cellular behaviour such as the motility as the stiffer a material is the more easily force can be transduced through a migrating cell[234]. Cells attached to electrospun polymers containing RGD deformed the fibres by exerting a contractile force on the fibres[26]. It is likely that cells would be unable to migrate on these materials as applied force provides deformation of the substrate rather than cell movement. SEM images of the electrospun polymer before and after deformation by cells are shown in Figure 3-23.

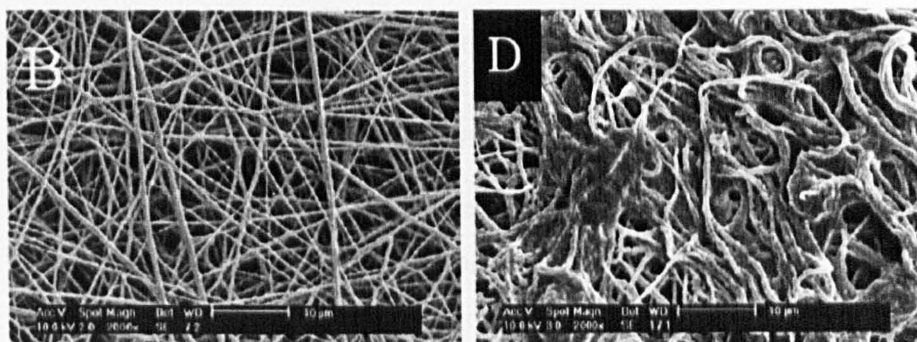


Figure 3-23 RGD-modified PLGA electrospun mesh (B) and the same mesh after 8 hours adhesion of fibroblasts (D). Reproduced from Kim et al[†,26].

Coating Effects

The background substrate on which a coating is formed may affect the accessibility or orientation of ligands and produce different cell behaviours for coatings of the same peptide on different backgrounds. When the hepatocytes were grown on coatings of a 2.3kD peptide on different types of polystyrene it was shown that the type of polystyrene that the coating was formed upon altered the response of the cells from a growth mode to a differentiated phenotype both in morphology and secretion of DNA or albumin. The authors believe that the alteration in behaviour may be caused by different orientation and conformation of the peptide on the different plastics[93].

† Reproduction of Figure 1 and Figure 6 from "Kim, T. G. & Park, T. G. Biomimicking Extracellular Matrix: Cell adhesive RGD Peptide Modified Electrospun Poly(D,L-lactic-co-glycolic acid) Nanofiber Mesh. *Tissue Engineering* **12**, 221-233 (2006)" with permission from Liebert Publishing.

When streptavidin engineered to contain peptide sequences from fibronectin or vitronectin was coated on polystyrene or biotin-BSA the levels of cell adhesion were reduced on the biotin-BSA coatings especially at low coating concentrations and a greater dependence on the peptide sequence was observed[53].

3.4.1.3 Comparison of Synthesis Methods

Coating vs. Modification

Poly(Nipaam-co-NASI) have been prepared and either reacted with the peptide and then deposited as a film or the film prepared first and the peptide subsequently reacted with the film[73]. It was found that the first method (coating) did not promote cell adhesion whereas the second method (modification) did. The authors assumed that this was because the majority of the ligands were buried in the bulk-phase of the film when the coating was formed after the attached of the peptide.

Irvine et al produced combs that were modified in solution (followed by coating), activated in solution and coupled in situ (modification procedure), or both activated and coupled in situ (modification procedure). They showed that the levels of peptide immobilisation were 30% lower by the last method than the others (see section 3.2.2)[79]. The amount of peptide contained in the final substrate was reflected in the adhesion of cells to the substrates prepared by the various methods. There was no reduction in the amount of cell adhesion on peptide containing coatings that had been cast onto a preformed solid indicating that peptides were accessible towards ligands which has been attributed to a quasi-2D confinement of the comb polymer at the water interface to minimise interaction of water with the backbone and maximise interactions with the chains.

Similar levels of cell adhesion were observed on substrates of BSA-RGD regardless of whether the protein-peptide conjugate was synthesised in solution and adsorbed or the protein was adsorbed first and then the peptide covalently attached in situ[43].

A study of focal adhesions on cells adhered to fibronectin coated or fibronectin immobilised surfaces showed two distinct types of cell-matrix adhesions[235]. On coated surfaces the cells formed "fibrillar adhesions" enriched

in tensin but lacking paxillin and vinculin. On the surfaces where fibronectin was covalently linked to the material the cells formed "classical" focal contacts containing high levels of paxillin and vinculin. The study associated the change in focal adhesion type with the rigidity of the substrate although by this they probably meant that the binding ligand was rigidly fixed to the substrate and unable to move.

Incorporation vs. Modification

Acrylamide gels have been prepared with varying concentrations of RGD via the copolymerisation of either a PCS or an NHS activated ester that was later displaced by the peptide[68]. Similar levels of RGD concentration were immobilised into the gels by both methods however the cellular response was dependent on the synthesis method. When the peptide was copolymerised the numbers of cells adhering to the surface were approximately half the numbers attached to the gels with equivalent peptide concentrations but synthesised via a modification route. This may be due to the localisation of peptide at the surface with the modification route but this seems unlikely as the activated ester will have been copolymerised throughout the bulk and the gels should be porous enough to allow access of reagents. It appears that the copolymerisation of a peptide adversely affects the activity of the peptide, possibly through reactions of the side chains of the peptide.

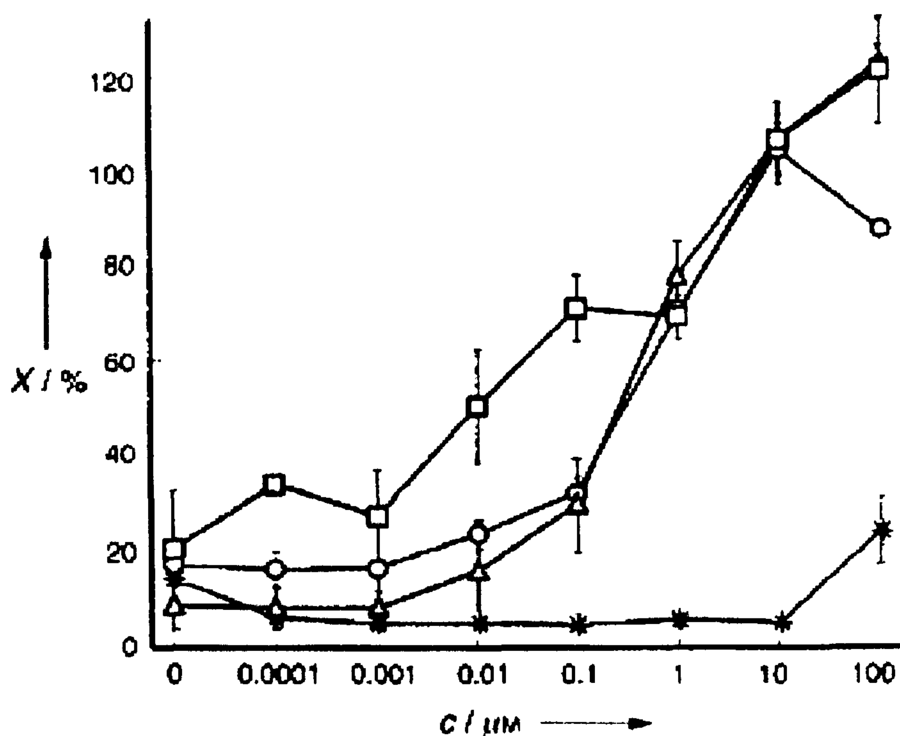
3.4.1.4 Distance of Peptide from Bulk

It is known that to achieve the promotion of cell adhesion the ligands must be spatially accessible towards ligands. This forms the requirement for a certain minimum spacing between the bulk of the material and the active peptide sequence. The distance can be achieved via additional amino acids in the peptide chain or through the incorporation of a "spacer arm". The distance that the peptide is required to be from the bulk material depends on the physical properties of the bulk such as openness of a network or water content in swollen materials that affect the accessibility of the bulk.

It has been shown that PEG-DA based materials synthesised by incorporating a peptide require a long spacer arm between the bulk and the peptide in order to promote cell adhesion[41]. This is not surprising as the PEG-DA contains two acrylate groups and hence forms a highly cross-linked network with

long chains extending from the backbones in a manner similar to a brush polymer. A peptide incorporated into the backbone by polymerisation would be buried inside the brush unless it was itself attached to a long chain. An interesting study was made on the accessibility of IgG attached to a PEG spacer arm in hydrogels composed of PEG-DA and poly(propylene fumarate-co-ethylene glycol) copolymers with varying molecular weights (CP 2000 and CP5000)[236]. The results showed in all cases a surface concentration lower than that predicted from the bulk measurements with the longer copolymer tending to reduce the amount of available ligands. Similar materials utilising PEG but with a modification method do not require a spacer arm because the immobilisation occurs only at accessible sites[95,101-104].

In a system that utilised the residual double bond in poly(methyl methacrylate) to polymerise a coating of acrylate-containing RGD onto the surface it was found that the shortest spacer arm tested did not effectively promote cell adhesion except at very high concentrations as shown in Figure 3-24 below[77]. The longer spacer arms were all equally effective at promoting cell adhesion.



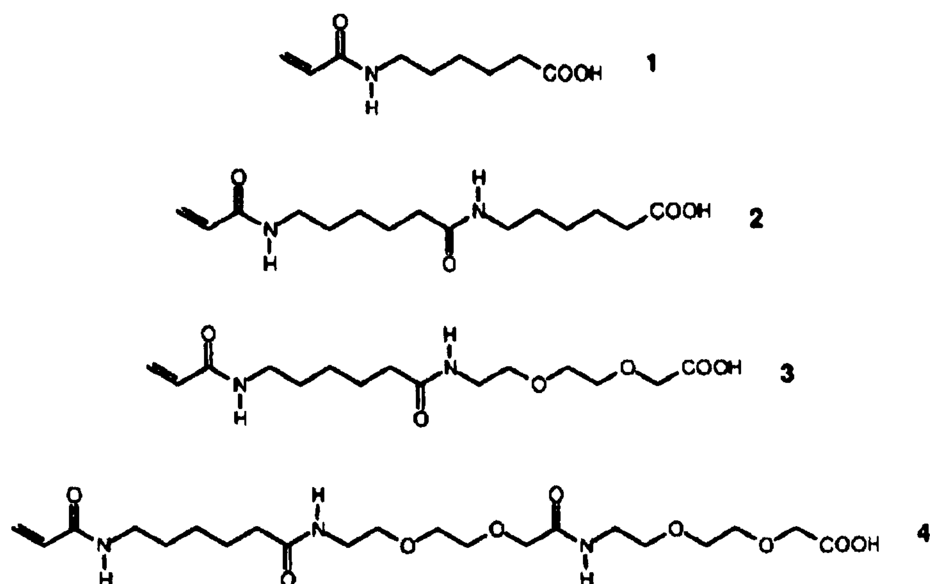


Figure 3-24 Cell plating efficiencies of osteoblasts on P~MMA modified with RGD-acrylate. Acrylate spacer 1 (star), acrylate spacer 2 (circle), acrylate spacer 3 (square, acrylic spacer 4 (triangle). Reproduced from Kantlehner et al[†,77].

† Reproduction of Figure 2 from "Kantlehner, M., Finsinger, D., Meyer, J., Schaffner, P., Jonczyk, A., Diefenbach, B., Nies, B. & Kessler, H. Selective RGD-Mediated Adhesion of Osteoblasts at Surfaces of Implants. *Angew. Chem. Int. Ed.* **38**, 560-562 (1999)" with permission from Wiley-VCH.

Beer et al synthesised G_n-RGDF with 1 to 19 glycine spacers and coupled these peptides to polyacrylonitrile beads with efficiencies of ~74% for 1-9 glycines and ~91% for 10-19 glycines[19]. The agglutination of platelets was assessed and it was found that as the length of the spacer arm increased up to 9 glycine residues the agglutination increased however as the length was increased further the agglutination dropped off. In competition with soluble peptide the concentration of the soluble RGD required to abolish agglutination was higher for the longer spacer arms, 41μmol for n = 1, 400μmol for n = 3 and 4mmol for n > 7. The decline of agglutination at longer spacer arm lengths is probably due to the increasing entropy with the flexible arms that prohibits strong binding to integrins.

The crystal structure of the $\alpha_v\beta_3$ integrin has been determined[237] and the binding site for RGD peptides identified[238]. From these it appears that the binding site is located very close to surface of the integrin and that long spacer arms should not be required for access as shown in Figure 3-25. It appears that the spacer arm is required to prevent steric hindrance and adverse electrostatic effects between the integrin and the bulk of the material. This would explain why the length of the spacer arm required is substrate specific.

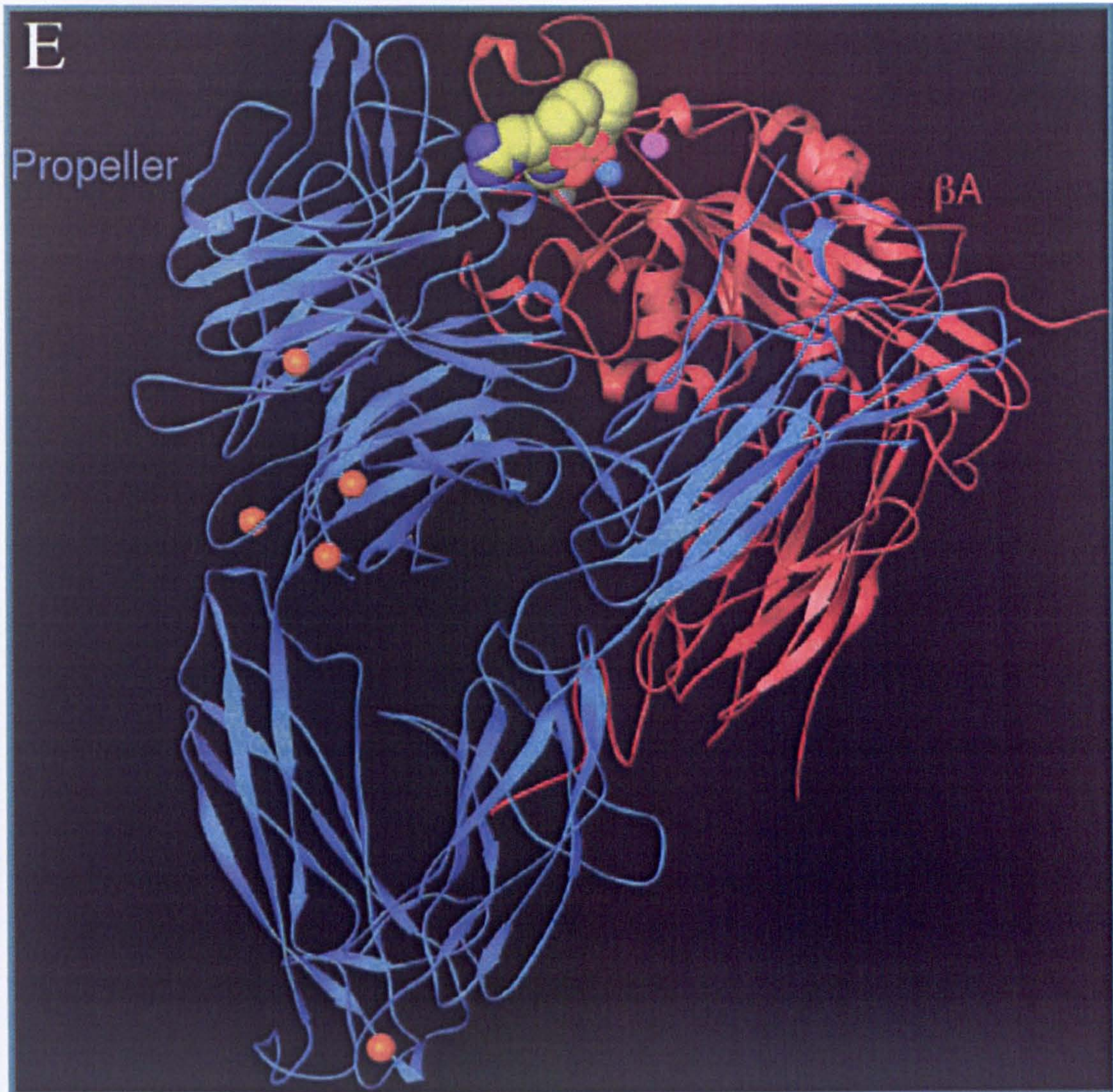


Figure 3-25 Structure of $\alpha_v\beta_3$-Mn complexed with cyclo(RGDf-N(Me)V). Ribbon drawing, the carbon, nitrogen and oxygen atoms of the cyclo-peptide are shown in yellow, blue and red respectively. Reproduced from Xiong et al[238,†].

† Reproduction of Figure 1.from "Xiong, J.-P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L. & Arnaout, M. A. Crystal Structure of the Extracellular Segment of Integrin αvβ3 in Complex with an Arg-Gly-Asp Ligand. *Science* **296**, 151-155 (2002)" reprinted with permission from .AAAS

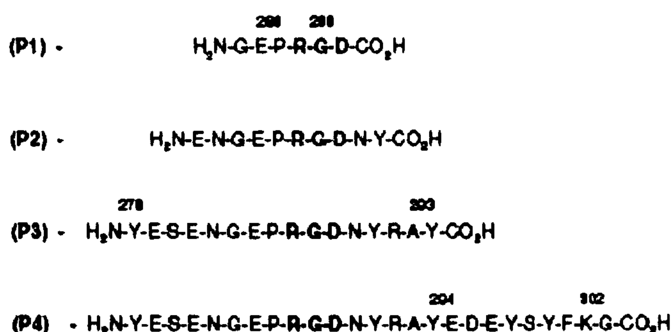
3.4.2 RGD Sequence

A large number of different sequences and lengths of RGD-containing peptides have been utilised to form biomaterials, these range from the minimum sequence RGD to entire proteins. Much of the analysis of the binding affinity of various RGD containing peptides has been performed on the detachment of cells from coatings of fibronectin or vitronectin by the soluble peptides[161,239,240]. It has been shown through these methods that the relative affinities of a series of short peptides for the fibronectin integrin receptor went in the order GRGDSP>RGDS>RGD-NH_2>RGD[241]. In this study the minimal sequence RGD had very little ability to detach cells from fibronectin coatings. In addition it was shown that GRGDPPC had very little ability to detach cells from fibronectin or vitronectin coated surfaces but GRGDSPC and GRGDNPC were active on both coatings but had increased preferences for the vitronectin and fibronectin integrins respectively. This shows that, as well as the length of the peptide sequence, the flanking amino acids around the RGD peptide can alter the affinity and selectivity of the sequence.

Despite the reported inability of the minimal RGD sequence to bind to integrins there are a number of studies that have utilised this sequence and shown improved cell attachment[54,59,65,67,169], spreading[57,227], attachment strength[176], or viability and proliferation[63] although it is likely that more significant improvements could have been obtained with more affine peptides. The ability of the minimal sequence to bind to integrins when immobilised on surfaces but not in solution is probably due to increased binding energy in immobilised peptides due to entropy loss on binding being minimised when the peptide is covalently linked to substrates. The effect of entropy on the binding abilities of peptides has also been observed when RGD-containing peptides have been linked to long flexible polymer chains (see section 3.4.1).

3.4.2.1 Length of Peptide Sequence

Rapuano et al. showed that the fibronectin fragments P2 and P4 (shown below) adsorbed to polystyrene in equivalent molar amounts such that each coating presented the same number of active moieties[90]. The fragments were systematically chosen such that the longer peptides contain the same sequences of amino acids as found in the shorter peptides. It was found that the longer peptide sequence P3 and P4 promoted a higher degree of cell adhesion indicating that flanking residues at some distance from the RGD sequence can affect the strength of binding to integrins, probably through effects on the conformation of the tripeptide. The peptide sequences and their ability to promote cell adhesion when utilised as a coating are shown in Figure 3-26 below.



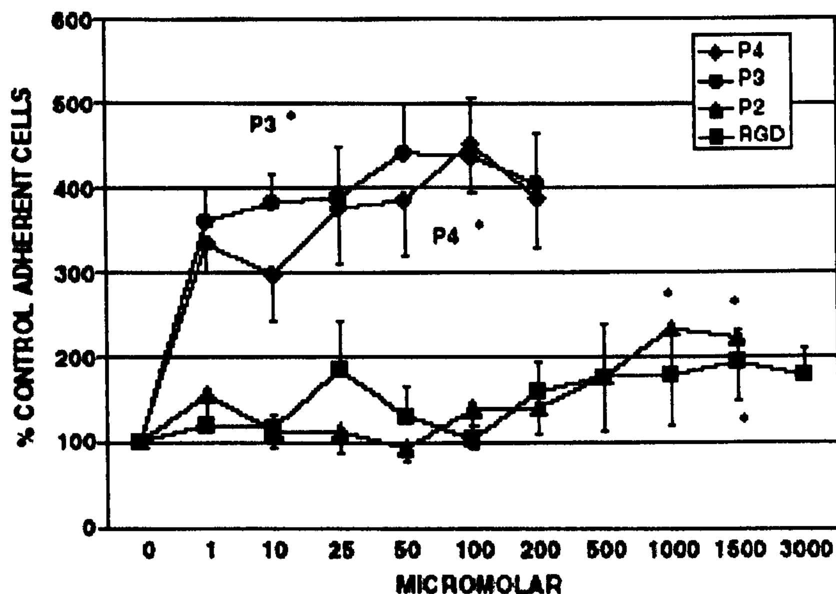


Figure 3-26 Adhesion of osteoblast-like cells to coatings of various peptides (shown to the left). Reproduced from Rapuano et al[†,90].

† Reproductions of Figure 1 and Figure 3 from "Rapuano, B. E., Wu, C. & MacDonald, D. E. Osteoblast-like cell adhesion to bone sialoprotein peptides. *journal of Orthopaedic Research* **22**, 353-361 (2004)" with permission from.John Wiley & Sons

Lanza et al created an immunoglobulin human/mouse chimeric heavy chain gene with a single or three repeat RGD tripeptide sequence inserted in the third hypervariable loop termed γ1RGD and $\gamma 1(RGD)_3$ antibodies[242]. Melanoma, osteosarcoma and fibroblastoma tumour cells did not adhere to coatings of the γ1RGD but did adhere to $\gamma 1(RGD)_3$ coatings. It is unlikely that the adsorption of the large gene will be affected by the length of the inserted sequence and so this shows that the repeat RGD sequence either provides more accessible active moieties or has a greater affinity for the osteosarcoma and fibroblastoma integrins.

3.4.2.2 Affinity

The flanking residues surrounding an RGD sequence can alter its conformation and affinity for various integrin receptors. A bent conformation has been shown to be more selective for the $\alpha_v\beta_3$ receptor. Lin et al modified polyurethane with GRGDVY and GRGDSY at the same concentration of 100 μmol/g of polymer[61]. When endothelial cells were grown on the polymers the GRGDVY peptide promoted significantly higher levels of cell adhesion than the GRGDSY peptide. This demonstrates the higher affinity of the former for the integrins expressed by the endothelial cells. Cyclic peptides have been shown to have a greater affinity for integrins than linear peptides. Linear peptides and cyclic RGDfK have been compared and the cyclic peptide shown to provide a greater increase in cell attachment when immobilised[201] and to be more effective at detaching cells from RGD-modified surfaces when in solution[243]. This was confirmed by Verrier et al who also showed that an increase in cell adhesion to cyclo-DFKRG modified surfaces compared to GRGDSPC over a range of peptide concentrations[92].

3.4.2.3 Integrin Selectivity

In addition to having a greater affinity for integrins some cyclic peptide have also been shown to selectively bind to certain integrins and not others. The selectivity is obtained via control of the peptide conformation through cyclisation and by utilisation of flanking residues that increase binding to the integrin in question.

The cyclic peptide cyclo(-RGDfK) has been shown to selectively bind to the integrins $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins expressed by osteoblasts[77,243,244]. The peptide's affinity for $\alpha_v\beta_3$ is 10-fold greater than that of the analogous linear peptide[245,246]. When cyclo-DFKRG was compared with GRGDSPC it was shown that the adhesion of osteoprogenitor cells to the cyclic peptide was inhibited by monoclonal antibodies to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ whereas the linear peptide was inhibited by β1 antibodies as shown in Figure 3-27[92].

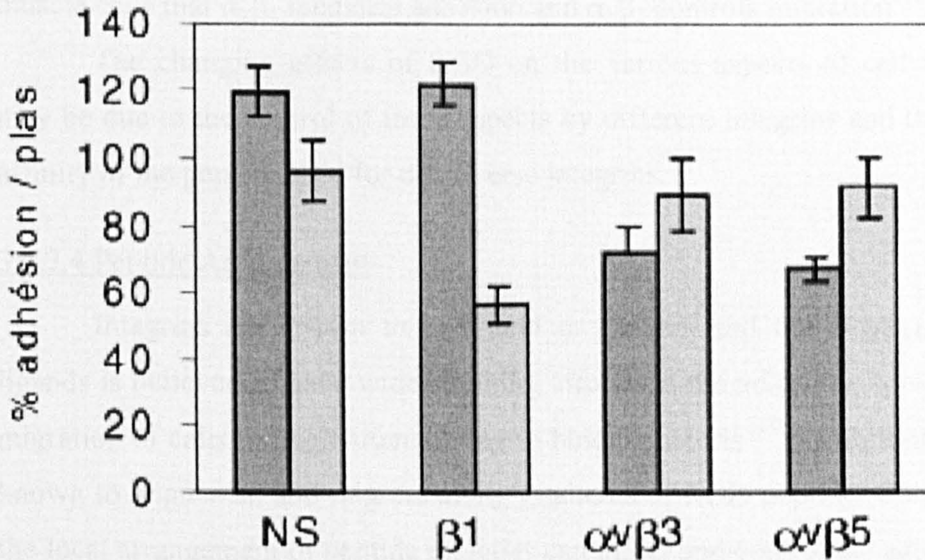


Figure 3-27 Inhibition of osteoprogenitor adhesion to surfaces modified with cyclo-DFKRG (dark grey) or GRGDSPC (light grey) by monoclonal antibodies to integrin subunits. Reproduced from Verrier et al[†,92].

The study also showed that the expression of integrins was altered by the nature of the peptide surface on which the cells were plated. GRGDSPC coating appears to reduce the expression of β_1, β_5 and α_5 subunits, while cyclo-DFKGR promoted the α_v and α_5 subunits, β_1 mRNA expression being slightly reduced and β_3 was poorly expressed on both surfaces. The regulation of integrin expression by the substrate on which cells are cultured has significant implications on the use of RGD in tissue engineering. Cells may be encouraged to produce the integrin that is most affine for the peptide utilised.

† Reproduction of Figure 7a from "Verrier, S., Pallu, S., Bareille, R., Jonczyk, A., Meyer, J., Dard, M. & Amedee, J. Function of linear and cyclic RGD-containing peptides in osteoprogenitor cells adhesion process. *Biomaterials* **23**, 585-596 (2002)" with permission from Elsevier Ltd.

Massia et al found that the binding of fibroblasts to GRGDY-modified glass was mediated primarily by the $\alpha_v\beta_3$ integrin, a vitronectin receptor, rather than the $\alpha_5\beta_1$ integrin (fibronectin receptor)[129]. It has been shown that melanoma adhesion to streptavidin engineering with RGD sequences was also mediated by the $\alpha_v\beta_3$ integrin[53]. It has also been shown that cyclic peptides with the general formula RGD-Ar-R (with Ar being a hydrophobic residue) have high affinity and selectivity for the platelet integrin $\alpha_{IIb}\beta_3$[240]. It has been shown with smooth muscle cells that $\alpha_v\beta_1$ mediates adhesion and $\alpha_v\beta_3$ controls migration[219].

The changing effects of RGD on the various aspects of cell behaviour may be due to the control of these aspects by different integrins and the relative affinity of the peptide used for the diverse integrins.

3.4.2.4 Peptide Arrangement

Integrins are known to be found in clusters and the clustering of the ligands is believed to have wide-reaching effects on the adhesion, spreading and migration of cells through altered integrin binding effects[247]. In addition cells are known to align with and migrate along gradients of RGD peptide. Consequently the local arrangement of peptide moieties can affect and control the adhesion and migration properties of cells on peptide-containing materials.

Clustering

Albumin derivatised with 3.5-20.8 RGD moieties per protein molecule and coated in varying concentrations onto TCP has been used to create surfaces where the total amount of RGD moieties was constant but the distances between active groups varied. It was found that the higher numbers of peptide moieties per albumin molecule were more effective at promoting cell adhesion[42]. This implies that the distance between moieties may be more important that the actual peptide concentration.

Star shaped polymers have been employed to form RGD clusters that were blended with non-derivatised polymers to form surfaces with equivalent overall densities of RGD but different localised densities. It was found that clusters of ligands increased cell adhesion, motility and the formation of stress fibres compared to materials with the same peptide concentration[208].

Lee et al formed alginate hydrogels from a blend of alginate and alginate modified with varying numbers of RGD moieties per molecule and consequently varied both the overall ligand density and the spacing between clusters of ligands[45]. It was shown that the proliferation of preosteoblasts was linked to the spacings between islands of RGD and not to the overall RGD concentration or number of RGD ligands in each cluster. In contrast to this the secretion of osteocalcin (an osteogenic bone marker) was increased on materials with smaller cluster spacings but the same overall RGD density (i.e. smaller numbers of ligands in each cluster and less distance between clusters).

Gradients

Brandley et al created acrylamide gels with RGD-peptides incorporated in either uniform concentrations or gradients and showed that the uniform concentrations of peptide all promoted the same level of cell adhesion[69]. However when plated onto gels containing a gradient of RGD (linear or exponential) cells migrated along the gradient towards the higher peptide concentration.

3.4.3 RGD Concentration

3.4.3.1 Effect on Cell Adhesion and Spreading

It has been definitively reported that "An RGD Spacing of 440 nm is Sufficient for Integrin $a_v\beta_3$-mediated Fibroblast Spreading and 140 nm for Focal Contact and Stress Fiber Formation" by Massia et al in 1991[129]. This study derivatised glass with GRGDY at concentrations varying from 0.1-100 fmol/cm^2. The study showed that a concentration of 1 fmol/cm^2 promoted cell spreading and a concentration of 10fmol/cm^2 also promoted the formation of focal contacts (RGD spacing of 440nm and 140nm respectively).

A logarithmic relationship between the RGD concentration and the average cell area with increasing RGD concentration was shown by Reinhart-King et al who derivatised polyacrylamide gels with YAVTGRGDS in concentrations ranging between 0.001 and 1 mg/ml of peptide (approx. 1.2x10^{-3}-1.2 μmol/cm^2 assuming a surface depth of 10nm)[175]. There was no indication that the average cell area had reached a maximum over the concentration range tested. In addition to the cell area increasing with RGD concentration the morphology of the cells changed to include pseudopodia and present a stelate contour.

The average cell area was also found to increase with increasing RGD density in poly(vinyl alcohol) hydrogels[86]. At the maximum concentration of 2.8μmol per ml of polymer (equivalent to 2.8pmol/cm^2 assuming an accessible surface depth of 10nm) there was no indication that a maximum cell area had been obtained.

Drumheller et al modified a non-adhesive semi-interpenetrating network formed from PEG-DA, TMPTA and acrylic acid with GRGDS and found that concentrations of 66 and 110 pmol/cm^2 promoted 100% cell adhesion and that 12 pmol/cm^2 promoted up to 83% cell adhesion[40]. Another system using PEG, this time as a diacrylamide, studied the areas of cells adhered to RGD-containing hydrogels and found that the cell area increased with peptide concentration[96]. However the cells were not as fully spread as on TCP at the maximum concentration investigated was 3.8pmol/cm^2 implying that further improvements in cell spreading could have been made by larger peptide concentrations.

The concentrations required to achieve maximal cell spreading in these systems are much greater than those reported by Massia et al. This may be in part due to differences in experimental parameters such as cell type and peptide sequence utilised but part appears to be due to the nature of the material into which RGD is included. This may be because part of the cells' ability to spread comes from the background material or because the ability of the RGD peptide to function effectively is altered by the substrate on which it is immobilised. The PEG chains, which resist protein adsorption, may have sterically hindered the binding interactions between peptides and integrins thus requiring a much larger nominal peptide concentration to achieve the same perceived concentration. Alternatively the flexibility of the polymer chains to which the RGD peptides are attached may have reduced the binding strength of the peptide to the integrin through entropic considerations.

In addition the study by Massia et al did not investigate the cell density or attachment efficiency onto the surfaces. Since this work these parameters have become the main methods that have been used to quantify the efficacy of RGD-containing biomaterials (see Section 3.3.3). Observation of cell spreading and quantification of cell numbers may lead to different reported effectiveness of a given RGD concentration. Senyah et al investigated a similar system to Massia et al where GRGDSP was immobilised on borosilicate or titanium surfaces. A peptide input concentration of 40nmol/cm^2 obtained the best adhesion in terms of cell number.

Herbert et al found that a plateau of maximal cell attachment was achieved with concentrations of KGYSGRGDSPAS of 1pmol/cm^2 or greater immobilised onto ethylene glycol SAMs[195]. It seems likely that the long length of the peptide sequence has increased the binding affinity for integrins and a smaller amount of peptide is therefore needed to obtain maximal cell response.

Pakalns et al found that self-assembling monolayers containing 5 to 100 mol % of RGD-containing amphiphiles produced a linear increase in the number of attached cells (note: 50 mol % = 94.5pmol/cm^2)[169]. However it has been shown that the cell number is not necessarily an indication of the degree of cell spreading as Roberts et al reported similar numbers of cells on self-assembling monolayers containing 10^{-5} to 1 mole fractions of RGD but the morphologies of the cells were very different as shown in Figure 3-28 below[248].

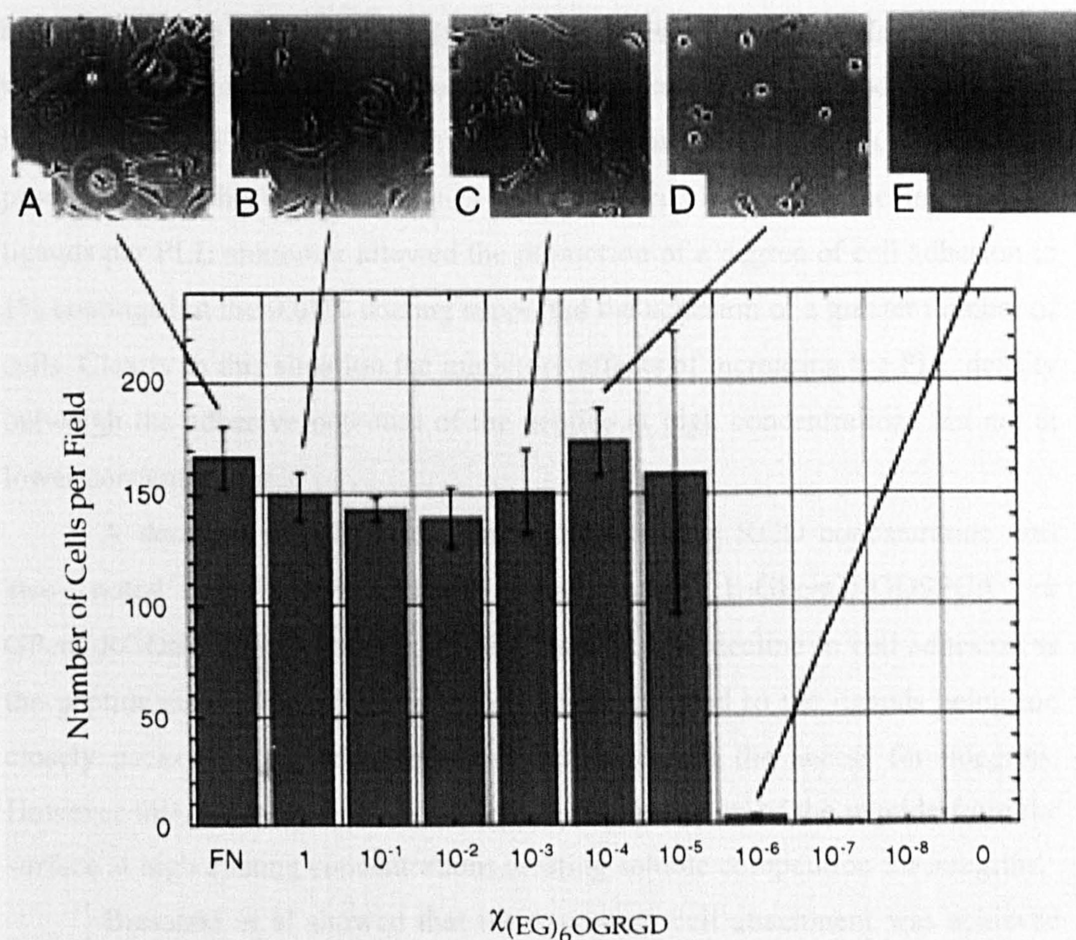


Figure 3-28 Adhesion of endothelial cells to SAMs presenting RGD and ethylene glycol groups. Reproduced from Roberts et al[†,248]

† Reprinted with permission from "Roberts, C., Chen, C. S., Mrksich, M., Martichonok, V., Ingber, D. E. & Whitesides, G. M. Using Mixed Self-Assembled Monolayers Presenting RGD and $(EG)_3OH$ Groups to Characterize Long-Term Attachment of Bovine Capillary Endothelial Cells to Surfaces. *Journal of the American Chemical Society* **120**, 6548-6555 (1998)" Copyright 1998 American Chemical Society

An increase in peptide concentration is not always guaranteed to increase cell adhesion and spreading as shown by Yang et al who found the greatest cell spreading when the minimum concentration investigated of PLL-GRGDS (0.03μM) coated onto PLA was utilised[56]. The coating of PLL-GRGDS significantly increased the average cell area when applied in concentrations of 0.3 and 0.03mM but not at the higher concentrations of 3 and 30μM. It seems likely that an improvement over the PLA could be obtained by the use of RGD but that the use of PLL in peptide-containing species meant that the active moieties were blocked when large coating concentrations were used. In a similar investigation[60] it was found that coating concentrations of 1% (w/v) did not promote cell adhesion but coatings of 0.01% did. Increasing the numbers of ligands per PLL monomer allowed the promotion of a degree of cell adhesion to 1% coatings but the 0.01% coating supported the adhesion of a greater number of cells. Clearly in this situation the inhibitory effects of increasing the PLL density outweigh the adhesive potential of the peptide at high concentrations but not at lower concentrations.

A decrease in cell attachment with increasing RGD concentration was also noted by Sawyer et al who coated E_7GPenGRGDSPCA or GPenGRGDSPCA onto hydroxyapatite disks[165]. The decline in cell adhesion as the peptide concentration increases has been attributed to the ligands being too closely packed at high concentrations and hindering the access for integrins. However this might also be attributable to de-adsorption of the peptide from the surface at high coating concentrations creating soluble competition for integrins.

Biesalski et al showed that the maximum cell attachment was achieved with self-assembling monolayers containing 10 mol % of the RGD-containing species[172]. This is in contrast to the report by Pakalns et al discussed earlier where the increasing mole percentage of the peptide in self-assembling monolayers increased the cell adhesion up to 100 mol %. It is known that packing effects can trouble SAMs at high peptide concentration and it seems likely that the reduction in cell attachment at high concentrations is in fact due to lowered accessible ligand concentrations.

3.4.3.2 Effect on Viability and Proliferation

Some researchers have found that the inclusion of RGD reduces the rate of proliferation or even decreases the viability of cells over time (see section 3.3.1 Cell Behaviour). This has also been observed at varying concentrations of RGD as shown by the relative numbers of fibroblasts on keratin and keratin-RGD coatings of different concentrations after 1 hour and after 24 hours[52]. Initial cell attachment to coatings of 0.2-0.8mg/ml keratin-RGD were the same independent of the coating concentration. The relative number of cells on the peptide containing surfaces compared to glass after 24 hours is lower than at the 1 hour time point. Although cell numbers at the later time point were higher than that of glass the fact that the numbers of cells were closer to that of the control substrate than at an earlier time point indicates that the proliferative rate was less than that of glass. The extent of the reduction in proliferative rate was ameliorated to some extent by the utilisation of a stronger coating concentration but the use of the keratin-RGD peptide containing species appears to have had a negative effect on proliferation.

When proliferation has been examined as a function of the concentration an increase in RGD concentration tends to increase the growth rate[45,249-251]. Holland et al also found a clear link between the rate of endothelial cell spreading in the first hour of attachment and the rate of proliferation over 4 days as a function of the RGD concentration[189].

3.4.3.3 Effect on Motility

Cell migration occurs via the extension of lamellae from the cell followed by a contractile force exerted on the lamellae and the retraction of lamellae on the opposing side of the cell[252]. The formation and the retraction of the lamella are governed by integrin binding to the substrate. At low levels of cell attachment migration is slow because weakly attached cells do not generate enough traction to move significantly[234,253]. At high levels of cell attachment cells display impaired motility, presumably due to an inability to cycle between adherent and non-adherent states because the ligand binding is too strong. The levels of cell attachment and migration rates are dependent on the ligand density, ligand-integrin binding energy, integrin density and activation state of the integrins[254,255].

There is evidence to show that the migration of cells on RGD-containing surfaces is dependent on the RGD peptide as well as the integrin profile and is affected by the sequence utilised, the binding energy (affinity) and the concentration. The presence of RGDS has been shown to decrease the ability of cells to migrate in terms of random motility coefficient, migration differential or persistence time[127]. The highest migration rates have been found at intermediate peptide concentrations in polyvinyl amine coatings[249,251] and through PEG-DA hydrogels[98]

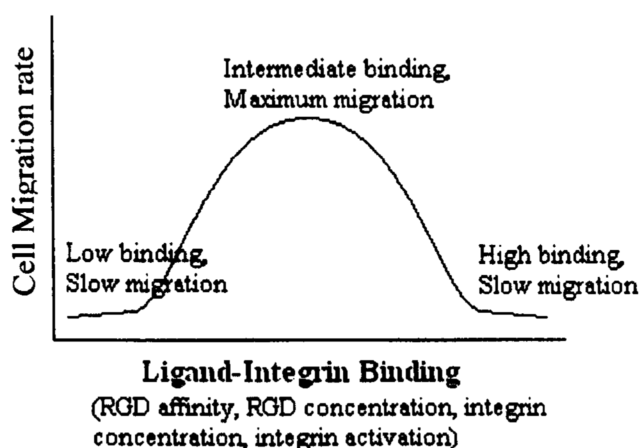


Figure 3-29 Theoretical bell curve for the dependence of cell migration rate on ligand-integrin binding.

A theoretical bell-curve graph such as that shown in Figure 3-29 has been experimentally proven to fit the motility of cells on collagen with respect to the amount of GRGDSPC present[28]. In contrast Maheshwari et al found a plateau at which cell speed stabilised and did not decrease on substrates formed from clusters of PEO-YGRGD[28]. The cell speed was dependent to a greater extent on the cluster sizes rather than the overall average density of peptide indicating a cooperative effect of clustered ligands on the assembly of regulatory and structural proteins. It is possible that the cell speed would decrease if the peptide concentration was increased further. However Maheshwari et al estimate 100 integrins/μm^2 compared to 200,000 peptide moieties/μm^2. It appears that the low affinity of the peptide YGRGD for integrins results in a modified migration profile wherein the cells are always able to de-adhere sufficiently to migrate.

3.4.3.4 Extracellular Matrix Production

Rezania et al found that extracellular production was increased on glass surfaces derivatised with concentrations of 0.01, 0.62 and 3.8 pmol/cm^2 of CGGNGEPRGDTYRAY[125]. The matrix produced by osteoblasts was significantly improved over the RGE control for the 0.62 and 3.8 pmol/cm^2 concentrations. However the increase was the same for both of the higher concentrations implying an on/off relationship between RGD concentration and extracellular matrix production.

In contrast to this Mann et al found that ECM production was reduced on glass surfaces modified with RGDS (2nmol/cm^2) compared to RGES and non-modified glass even though the original cell adhesion was promoted[131]. It has been suggested that the RGD-ligand interactions may initiate signal transduction events to regulate ECM synthesis.

The adsorption of extracellular matrix produced by cells to surfaces is dependent on the nature of the surface. Roberts et al produced self-assembling monolayers of non-fouling ethylene glycol units and RGD that showed cell attachment and spreading but resisted the deposition of newly formed extracellular matrix[248].

3.4.3.5 Differentiation

RGD-modified surfaces appear to be able to promote differentiation of cells as shown by the secretion of various proteins. Increasing the concentration of a PronectinF coating upregulates the production of endothelin-1 by endothelial cells[214]. Dopamine production in pheochromocytoma cells is increased by increasing RGD concentration[178]. PEG-DA hydrogels containing varying concentrations of RGD were synthesised to encapsulate marrow stromal cells and the expression of bone related markers was examined[34]. Whilst the total DNA content of the hydrogels remained independent of the RGD concentration the expression of alkaline phosphatase and osteocalcin were increased as the RGD concentration increased showing that larger amounts of active moieties promoted differentiation towards osteoblast-like cells.

Connelly et al cultured bone marrow stromal cells on RGD and RGE modified alginate surfaces and attempted to differentiate the cells toward chondrocytes through use of dexamethasone and TGF-β1[256]. The differentiation

as determined by chondrocytic gene expression and matrix production was significantly inhibited by RGD but not RGE.

3.4.3.6 Other Aspects of Cell Behaviour

Schense et al measured the neurite outgrowth rates from dorsal root ganglia encapsulated in RGD-modified fibrin gels[27,35]. Intermediate RGD concentrations promoted the extension but higher concentrations inhibited the neurite outgrowth in a similar manner to that shown for migration of cells in other systems. The intermediate concentration at which growth was promoted was lower for a cyclic peptide than a linear peptide. This supports the theory that neurite outgrowth is governed by the same bell curve type behaviour as migration as the binding strength of cyclic peptides tends to be greater than the binding strength of linear peptides.

Reinhart-King et al showed that the cell area was proportional to the amount of peptide incorporated over the concentration range tested and that the endothelial cell traction forces generated were proportional to the cell area[175].

Ferris et al reported that the thickness of new bone formed in apposition to RGDC coated implants compared to plain titanium implants in rat femurs was 28% greater after 2 weeks and 45% after 4 weeks[118]. Schliephake et al found only limited evidence that RGD peptides linked to collagen coatings increased periimplant bone formation in the alveolar crest[257]. Woerly et al found that a poly(hydroxypropyl methacrylate) hydrogel containing RGD peptides was suitable for guiding the repair of spinal cord but the effects of RGD inclusion over a control substrate were not examined[83].

3.4.4 Other Peptide Sequences

In fibronectin the effect of the RGD peptide sequence is synergistically linked to the PHSRN site. When films of amphiphiles containing GRGDSP, GRGDSP and PHSRN, or sequences containing both moieties were constructed it was found that the inclusion of PHSRN increased initial cell attachment. The viability of cells on films containing only RGD or RGD and PHSRN at randomised distances declined over a 72 hour period whereas viability was maintained when RGD and PHSRN were contained in the same sequence with an $(SG)_5$ linker between the two active sequences[170].

Neurite outgrowth was measured by Saneinjad et al on glass/gold surfaces modified with 447 fmol/cm^2 of various adhesion peptides including CGRGDS[258]. Whilst RGD was shown to improve the number and length of neurites the other peptides SIKVAV and YIGSR were shown to be more effective at this concentration. Levasque et al modified dextran hydrogels with CRGD or a combination of CPDGYIGSR and CQAASIKVAV at concentrations of ~2μmol/cm^3 and found that the YIGSR / KVAV materials promoted slightly greater numbers of neurite and dorsal root ganglia cells attached than to RGD materials[259]. This is not particularly surprising as the RGD sequence utilised has a low affinity for integrins and is being compared to substrates with two active peptides.

Mann et al prepared glass surfaces modified with 0.5 or 2 nmol/cm^2 RGDS and cultured smooth muscle cells, endothelial cells and dermal fibroblasts on the surfaces. The numbers of adherent smooth muscle cells on the RGD-containing materials were not significantly different from the RGES control[131]. Both Santiago et al and Gumusderelioglu et al compared the modification of materials with the minimal tripeptide RGD and other proteins and peptide sequences and found that whilst RGD promoted cell attachment it did not perform better than IKVAV[59] or insulin[87]. It was shown in section 3.4.1.2 that the strategies of altering crystallinity in hydroxyapatite or including charged species into hydrogels have proved more effective than the incorporation of RGD-containing peptides.

3.5 Conclusions

There are numerous different ways to immobilise RGD containing peptides onto surfaces for the purposes of tissue engineering.

The amount of immobilised reactive moieties when formed by physical methods is dependent on the adsorption efficiency, the relative adsorption efficiencies of the peptide-containing species (PCS) and other species, the amount of PCS in solution compared to the surface area and the retention efficiency. However in all cases the amount of accessible peptide may not be the same as the amount of immobilised peptide. The accessibility of the peptide can be reduced by an adverse orientation and conformation of the peptide, the masking of the active moiety by other chemical groups and the embedding of ligands in the bulk where the access for integrins is hindered.

The amount of immobilised peptide formed by chemical methods of synthesis can be controlled by the relative amounts of input peptide or amounts of functional groups available for derivatisation compared to the non-active volume or surface area of the substrate. Reaction efficiencies can vary dramatically between the various synthesis methods and can also vary over a range of input peptide concentrations. The efficiency of a modification reaction and the depth of modification can also be dependent on the material being derivatised due to accessibility of reactive groups at the surface and in the bulk through penetration of reagents in porous materials. Overall the amount of immobilised RGD will be dependent on the synthesis method, input concentration of peptide, reaction efficiency and the volume of inclusion. The amounts of active moieties perceived by integrins will further be dependent on the accessibility of the peptides and the swelling characteristics of the material. It should be realised in all situations that the amount of input peptide does not always correlate to the amount of immobilised peptide and furthermore that the amount of active moieties perceived by integrins may not be the same as the amount of RGD present in the system.

The effectiveness of RGD-containing peptides to improve the cellular behaviour on a substrate is dependent, in part, on the background substrate. The chemical and physical properties of a material can affect the ability of the peptide to bind to integrin and transmit the effects of integrin binding into cellular

behaviour. The rigidity of a substrate affects the ability of a cell to transmit force through focal adhesion and consequently alters the migration abilities. The surface topography or crystallinity of a material can also affect the ability of RGD peptides to improve aspects of cell behaviour. The biological properties of the background material can affect the extent of improvement gained from the incorporation of RGD as the peptide-integrin based system may be in competition with other mechanisms of cell attachment and spreading.

The effectiveness of an RGD-peptide immobilised onto a surface on cellular behaviour is basically dependent on the strength and extent of integrin binding to RGD peptides. However this is controlled by a number of other variables. The affinity of RGD-containing peptides for integrins in general is controlled by the strength of electrostatic interactions and overall entropy change when the peptide binds to the integrin. The entropy loss on binding can be reduced by constraining the RGD sequence into an accessible conformation by selective use of flanking residues or cyclisation and will be increased by long flexible spacer arms. The binding strength can be increased by the selection of flanking residues or constrained conformation that is specific to a particular integrin. Binding strength can also be increased by clustering of ligands. The extent of integrin binding will be dependent on the concentration of ligands and the concentration and types of integrins. The concentration of ligands is dependent not on the amount of immobilised RGD-containing peptides but on the amount of these peptides that are available for binding in terms of accessibility. The concentration and the types of integrins available in a cell are dependent on the cell type, cell source and conditions the cells are cultured under. Most cells express more than one type of integrin capable of binding to RGD and the different integrins can control different aspects of cell behaviour. The integrin expression profile and the affinity of the integrins expressed for the particular peptide utilised will affect the various aspects of cell behaviour.

Whilst Massia et al set a benchmark by reporting that an RGD concentration of 10fmol/cm^2 was sufficient to promote maximal cell spreading and focal contact formation this has not been reproducible in other systems. The majority of other studies that have investigated the effects of increasing RGD concentration involve flexible polymeric systems and it seems likely that the increase in entropy loss upon binding of integrins has formed the need for a

greater RGD concentration to offset the decreased affinity. In addition the amount of accessible peptides in polymeric systems may not be the same as the nominal or found peptide concentrations. In polymeric systems maximal cell adhesion and spreading is usually observed at concentrations between 1 and 100 pmol/cm^2.

Virtually all of the literature concerning RGD-containing materials shows that the peptide improves at least one aspect of the cell behaviour that was examined. It has been shown in this review that some materials may produce significant improvement in certain cell responses and conditions but not others. Some literature that compares RGD-modified surfaces to other peptide derivatised materials reports no significant change in the cell response from the inclusion of RGD over the controls. For example Mann et al prepared glass surfaces modified with 0.5 or 2 nmol/cm^2 RGDS and cultured smooth muscle cells on the surfaces. The numbers of adherent smooth muscle cells on the RGD-containing materials and their extracellular matrix production were not significantly different from the RGES control[131]. It appears likely that there may be a considerable amount of research that has not been published wherein the RGD peptide has not been successful within the experimental parameters examined. This will limit the applications of RGD-inclusion for use in tissue engineering to situations where the experimental parameters determined by usage factors match those in which the peptide inclusion is successful. It has also been shown through comparison of RGD-incorporation with other strategies that the peptide incorporation may be less successful than other methods of promoting cell adhesion.

It appears that the majority of literature wherein RGD is unsuccessful at promoting cell attachment and spreading relates either to coatings[24,82], instances where a short peptide sequence with low integrin affinity is utilised or where a low concentration of peptide is accessible for interaction with integrins. However there is sufficient evidence to conclude that RGD-containing materials may not be able to promote long-term viability, proliferation and normal cell function without additional assistance from, for example, adsorbed proteins or cell-derived ECM.

In conclusion biomaterials designers should design RGD-containing materials with care as to the choice of peptide sequence, immobilisation strategy

and accessible concentration of the peptide. In addition researchers should be aware of the effects experimental variables can have on the reported success of RGD-incorporation and be aware of the limitations of RGD-incorporation as a strategy for promoting the various aspects of cell behaviour. Long-term use of RGD as a tool for tissue engineering will probably see the sequence being used in conjunction with synergistic sites, other active peptide sequences and other methods of promoting or directing different aspects of cell behaviour.

4 Experimental Design

As shown in the introduction there are numerous ways of creating RGD-containing biomaterials and the synthesis and analysis methods can have a large effect on the reported outcomes. Consequently it is important that the synthesis and analysis methods utilised are carefully selected to ensure that the results obtained do in fact answer the questions posed in the original aims. The selection of the methods utilised are therefore imposed by the criteria created by the objectives, which must be clearly identified in advance.

4.1 Objectives

RGD is a popular sequence used for derivatisation of biomaterials to promote cell adhesion and spreading through binding to integrins. The RGD peptide sequence binds to a wide range of integrins and its affinity for certain integrins can be increased by selection of flanking amino acids and cyclisation to promote integrin selective cell adhesion. The integrin selective binding of RGD can be useful in promoting the adhesion of selected cell types such as osteoblasts. However many cells do not express integrins that can be targeted with a selective RGD peptide. For the purposes of promoting non-integrin selective cell adhesion in tissue engineering there are strategies other than the incorporation of RGD peptides that can promote cell adhesion and spreading at a reduced cost. This leads to questions over whether the incorporation of RGD-containing peptides is useful in non-integrin selective cell adhesion.

The main aim of this research is to determine whether the incorporation of RGD peptides into polymeric biomaterials is a useful tool for promoting non-selective cell adhesion for tissue engineering purposes. To determine whether RGD-incorporation is a useful strategy in tissue engineering it is important to determine the experimental parameters under which the peptide functions and produces maximal beneficial cell responses. Consequently the main aim of this research becomes an investigation into the parameters that control the extent of effects caused by RGD-incorporation. This main aim can be divided into several more specific questions that, if answered, can help to determine the role of RGD incorporation in tissue engineering.

1. If the adhesion of cells to the substrate is non-integrin selective is there value to be obtained in combining the two strategies of supporting cell adhesion, namely in promoting cell adhesion by RGD and supporting normal cell behaviour by protein adsorption to the substrate?
2. Does the ability of RGD to promote cell adhesion hold in the case where protein adsorption is present, i.e. are the processes competitive or cumulative?
3. Does the use of RGD peptides in a non-specific manner confer any value on the substrate that is not obtainable by other methods?

4.2 Design Criteria

The answers to these questions can be obtained by the synthesis and analysis of a library of materials which is designed to show the effects of changing background substrate composition and RGD concentration. The design of this library should ensure the following design criteria are met to allow logical and meaningful conclusions to be drawn from the data:

1.) The library should contain materials that possess the following properties:

a) Does not adsorb proteins

b) Does not adsorb proteins and contains RGD sequences

c) Does adsorb proteins

d) Does adsorb proteins and contains RGD sequences

2.) The source of the ability to support cells should be elucidated:

a) The properties of materials that contain peptide should be as similar as possible to those that do not contain peptide.

b) The peptide used should be known to promote cell adhesion in at least one situation.

c) The minimal peptide concentration required to promote cellular properties should be determined.

d) The ability of a known concentration of peptide to promote cellular properties in different polymers should be determined.

3.) Experiments performed on the materials should:

a) Ascertain ability of polymers to support cell attachment

b) Ascertain ability of polymers to support cell viability

c) Ascertain ability of polymers to support normal cell growth and functions.

4.3 Experimental Design

As shown in section 4.1 there are many different ways to incorporate RGD peptides into polymeric substrates. The synthesis method used to create the polymers should reflect the requirements imposed by the design criteria.

The first criterion is that materials that do and do not adhere proteins and do and do not contain RGD should be synthesised. These materials should be synthesised in a manner as similar as possible to each other to ensure that features of the synthesis do not affect the analysis of the materials. Features of the synthesis that may affect different monomers in different ways such as the adsorption of coatings or the presence of additives should be avoided. Synthesis of random cross-linked polymer networks from vinyl-containing monomers provides an ideal method for the creation of materials with varying properties as they provide materials that are easily tunable and easy to handle and the processing variables are limited. Methacrylates make a good candidate for monomer choices as some have been approved by the federal drug agency (FDA, USA) and the medicines and healthcare products regulatory agency (MHRA, UK) for use in medical devices. In addition to this a range of methacrylates with different hydrophilicities are readily available. Polymers made from glycerol methacrylate are known to promote minimal cell attachment whereas polymers containing alkyl methacrylates (e.g. butyl, lauryl) are known to promote reasonable levels of cell attachment.

The requirement that the source of the ability to support cells should be elucidated means that the polymers containing RGD should be as similar as possible to the polymers without RGD. This means that additional functional groups such as acids or amines should not be included in the polymer to allow the attachment of RGD. The best way to incorporate RGD into a polymer without the unnecessary inclusion of functional groups is to copolymerise a vinyl-containing peptide with the other monomers. This also gives the added advantage that the peptide concentration can be precisely controlled to obtain various concentrations and also to ensure that different substrates contain the same RGD concentration.

Whilst the peptide utilised should be known to stimulate cell adhesion it should also have the least possible effect on the properties of the polymer. In many circumstances it has been found that there is a certain minimum distance between the peptide and the substrate that is required to allow the peptide to work. However this "spacer arm" may have a large impact on the properties of the polymer and so peptides with and without a spacer arm will be investigated. The minimal sequence needed to promote cell adhesion is RGD however the tripeptide has a much lower activity than slightly longer peptide sequences and the lower activity means it is unlikely to promote cell adhesion to a significant degree or that the concentration of peptide required to have a significant effect will be high. Consequently the peptide sequence GRGDS was chosen to be investigated. The reported minimal peptide concentrations required to promote cell adhesion on different backgrounds differs widely and it is not known what concentration is required to promote cell adhesion under these conditions. Consequently it was decided to investigate a range of different peptide concentrations over 4 orders of magnitude, these being 10, 1, 0.1 and 0.01 $pmol/cm^2$ (equivalent to 10, 1, 0.1 and 0.01 μmol/g assuming an integrin penetration depth of 10nm and a swollen hydrogel density of $1 g/cm^3$).

In vitro culture of cells is generally the first stage of analysing the efficacy of materials intended for tissue engineering purposes. Fibroblasts are a good choice of cell type for the first stage of analysing the material as they are easy to culture in large numbers and are able to show a large range of behaviour as they can survive in moderately hostile conditions. Primary human dermal fibroblasts were selected as the cell type for the analysis of the library of polymers as it is known that immortalised cell lines can respond differently to biomaterials than the primary cells that would be utilised in tissue engineering.

There are many different techniques that have been utilised to analyse the cellular functions on the materials under investigation. RGD acts by promoting the cell adhesion via integrins and the adhesion has many knock-on effects as anchorage-dependent cells cannot perform any other functions without adhesion. The quantification of the adhesion can therefore be performed by analysing a different function of the cells. It was decided to utilize the MTT assay, which is a calorimetric assay based on the purple colour from formazan formed from the

oxidation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as shown in Figure 4-1 below.

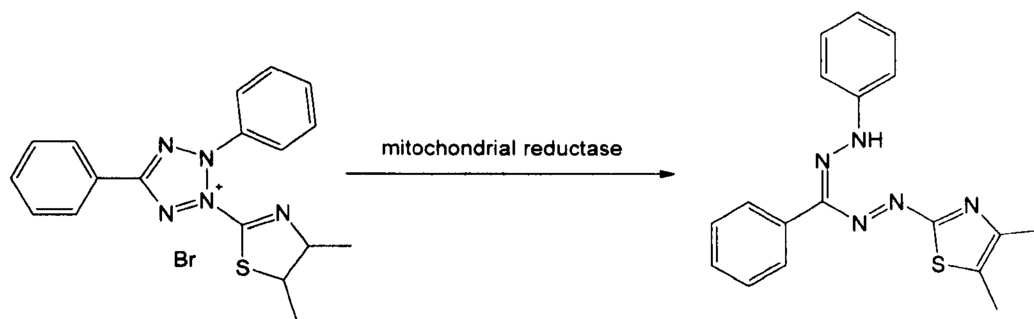


Figure 4-1 Oxidation of MTT by mitochondrial reducatase enzymes.

The MTT is oxidised when mitochondrial reductase enzymes are functioning and so the intensity of the formazan is directly proportional to the activity of these enzymes. Consequently the assay gives a response based on the cell number and viability thus generating a value that denotes both the number of cells present and their ability to function. It was also decided to visualise the cells to determine their morphology as abnormal morphology is an indication of cellular distress. The visualisation can be achieved by visual light microscopy or by immunofluorescent microscopy. The light microscopy can be performed at any time point but the contrast obtained is often not high enough to properly distinguish cell morphology. The RGD-mediated adhesion of the cells is known to have a large effect on the cytoskeleton and hence immunofluorescent staining for F-actin to visualise the cytoskeleton is the ideal method to investigate the morphology of cells adhered to RGD-containing materials.

5 Results & Discussion

5.1 Polymerisation Techniques

The first step towards the production of a polymer library was to was to investigate the polymerisation techniques that could be utilised to synthesise the biomaterials and investigate the effect of the synthesis method on the cell-based analysis of the materials. The requirements of this project were that polymers that both did and did not adsorb proteins should be synthesised. The simplest method of producing such substrates is to free-radically polymerise vinyl-containing monomers as these are cheap, easy to obtain in a wide range of properties and can easily incorporate cross-linkers. Additionally there is a large amount of published material on the biological properties of these types of polymers.

5.1.1 AIBN Initiation

Thermal initiation utilising AIBN at 60°C is a common technique that has been much utilised for the synthesis of biomaterials. Random cross-linked polymers can be synthesised by placing monomer solutions containing 1% AIBN into a mould followed by curing at 60°C. The resultant gel will be the same shape as the mould although its dimensions can alter if the solvent is changed reflecting the ability of a network to adsorb that particular solvent.

Hydrogels containing 2.5 wt % ethylene glycol dimethacrylate (EGDMA) and varying ratios of glycerol monomethacrylate (GMMA) and butyl methacrylate (BMA) were synthesised by thermal polymerisation in moulds. The moulds were created from 2 sheets of PET laminated onto glass with a PTFE gasket and held together with an aluminium frame that held the array together tightly through butterfly screws.

Moulds were made with a PTFE gasket of 1.5mm so that the needle diameter could be incorporated into the depth of the gasket and polymerisation occurred without significant monomer leakage. Sheets of poly(GMMA-co-EGDMA-co-BMA) were synthesised, cut into discs with a cork borer and analysed for their ability to support cells.

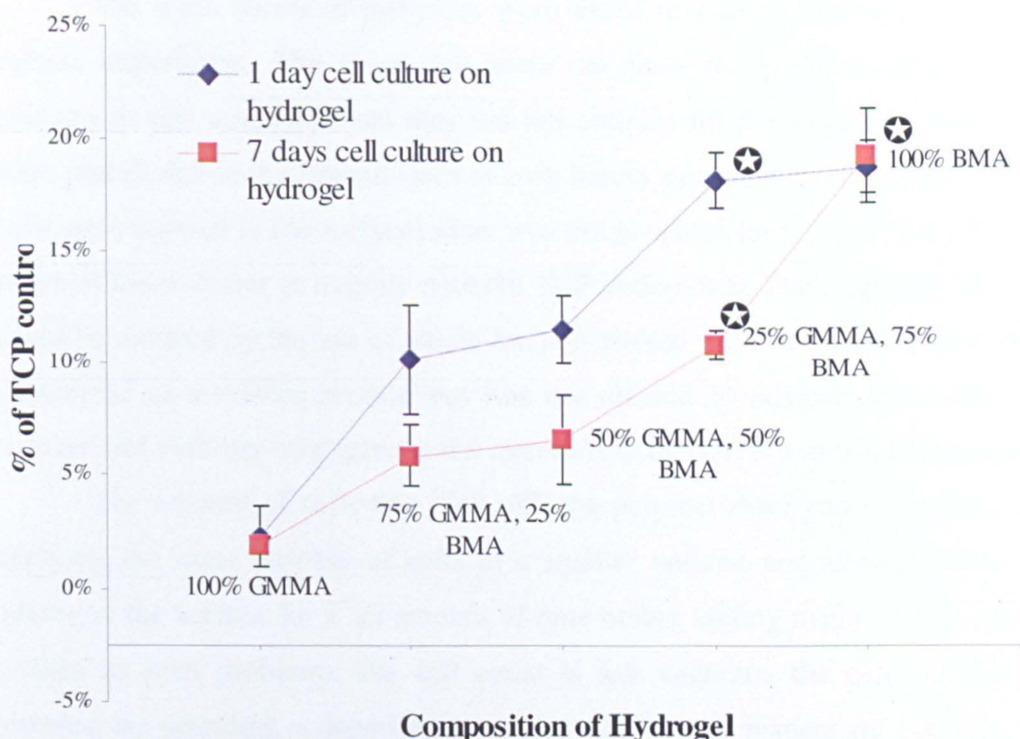


Figure 5-1 Human dermal fibroblast adhesion and survival on thermally initiated random copolymers of GMMA and BMA (percentages shown are wt%) containing 2.5wt % EGDMA. Cells (F1833, passage 7) were cultured on the substrates for 24 hours in media containing 10% FCS and were assessed by the MTT assay. Results shown are the mean of 3-6 replicates and error bars show standard deviation divided by number of replicates. Students non-paired T-test was performed on the data, all polymers were significantly different to TCP ($p<0.01$) and ✪ indicates that $p<0.01$ compared to 100% glycerol methacrylate hydrogels.

As shown in Figure 5-1 above the ability of these polymers to support cells increases as the percentage of BMA (the more hydrophobic component) increases. For the homopolymers of GMMA and BMA the relative viability of cells remains the same, proportional to the TCP control, over both the one and seven day time periods. The copolymers of GMMA and BMA appeared to be better at promoting initial attachment and viability of cells than at sustaining viability and promoting proliferation long term when compared to TCP. Viability and proliferation was observed on these hydrogels but this was not as pronounced as that produced on TCP resulting in the apparent decline of cell viability over 7 days cell culture in the graph above.

The thick sheets of polymers were found to cause problems in the cell culture experiment. The discs that were cut from the polymer sheets were uniform in size and shape but they did not entirely fill the wells in which they were placed due to the limited sizes of cork borers available. Consequently when cells were applied to the surfaces there was the potential for cells to "fall off" the edges of the polymer or migrate onto the TCP underneath. The migration of cells could be reduced by the use of sterile but non-treated multi-well plates. This was considered as a culture method but was not utilised as comparison of the cell number and viability on experimental materials with TCP is a useful benchmark.

The amount of cells that "fall off" the polymer discs can be reduced by applying the same number of cells in a smaller volume and allowing them to adhere to the surface for a set amount of time before adding media. This method creates its own problems, the cell count is less accurate, the cell viability is lowered by spending a significant amount of time in inadequate amounts of media and the space available to the cells is significantly affected by the wettability of the polymers. Even with this method the cells can be washed onto the TCP by the addition of media and the cells can also migrate onto the TCP. MTT assays of the wells in which the polymers were cultured showed that the cells attached to the TCP ranged from 20 to 90% that of the TCP control, indicating that significant amounts of cells had moved off the polymers and that the amounts varied indiscriminately between wells.

Another problem with this method of polymer synthesis was that large amounts of monomers and consequently large amounts of peptide are required to synthesise enough polymer to carry out the analysis. This method required 5g of monomers to produce enough surface area of one hydrogel type for one experiment. Due to the expense of the peptides and the importance of controlling the peptide concentration the reduction of monomer usage is important. As a consequence of this other alternatives for initiating the free-radical polymerisation were investigated.

5.1.2 UV Initiation

UV free-radical initiation has been extensively used for many years in coating applications however it is only in recent years that it has been used for biomaterials (see Section 3.2.3.1 Chain-growth Polymerisation). 2-hydroxy-2-methylpropiophenone (HMPP) has been approved by the FDA and the MHRA for use in biomaterials and so appears to be a good candidate for the photoinitiation of these materials.

5.1.2.1 Sheets

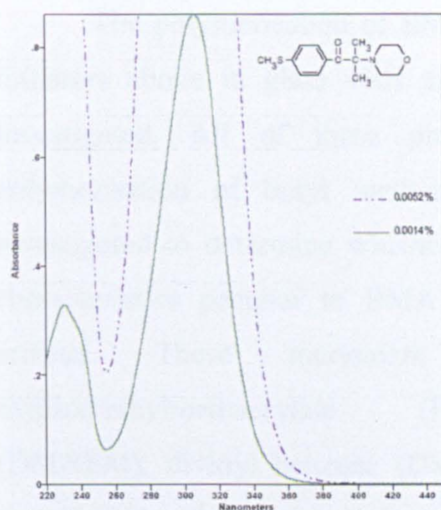
Monomer solutions were purged with nitrogen for a minimum of 20 minutes and then injected into a mould similar to that used for thermal initiation. Modifications that were made to the mould were to use quartz plates to allow passage of UV radiation, to use the thinner PTFE gasket and to leave a small gap at the top for injection. The gap at the top to allow injection was possible because the shorter polymerisation time involved with photo-initiation meant that solvent loss would not be significant and consequently much thinner sheets could be formed.

It was found that poly(GMMA-co-EGDMA) polymerised easily after 40 seconds exposure on each side. Polymers with 25 wt % BMA also polymerised but only after a further 40 seconds exposure. Polymer solutions with greater than 25 wt % BMA did not polymerise even after a total exposure time of 5 minutes. Greater exposure times were not investigated because the polymer mould became hot during 5 minute exposure times. It was believed that the lack of polymerisation in the BMA containing polymers was caused by air inhibition caused by the gap at the top and that this did not affect the GMMA polymers due to the faster polymerisation rate of these solutions.

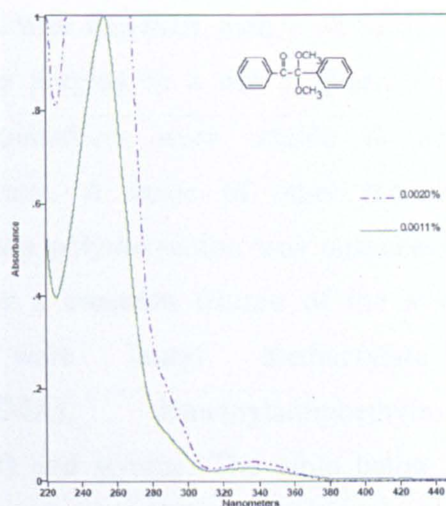
5.1.2.2 Formed in situ

The ideal situation would be for the polymers to be formed in sterile conditions in a container that could also be utilised for cell culture. Monomer solutions were placed in a 24 well culture plate and then placed inside a purging box where oxygen was removed by repeated cycles of applying vacuum and introducing nitrogen. Poly(GMMA-co-EGDMA) was synthesised in the wells of 24 well plates with 1wt % HMPP and 50 wt % ethanol. However it was found

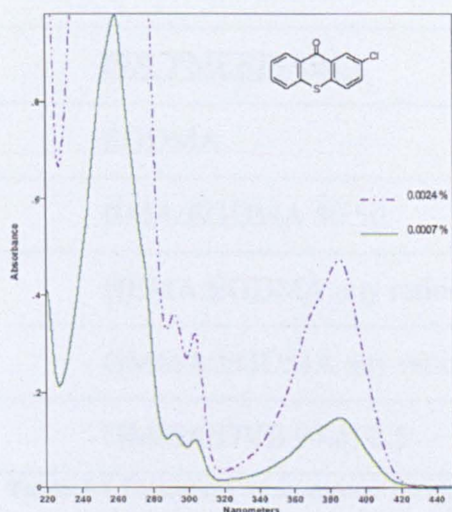
that compositions with more than 25% BMA degraded the plastic. This was the case with a number of different solvents (methanol, ethanol, IPA, DMSO) and also in the absence of solvent. In addition the mixture did not polymerise, it is possible to replace the plastic multiwell plates with glass multiwell plates if the polymerisation can be performed. It was believed that the lack of polymerisation of butyl methacrylate at high concentrations might be due to the absorption of UV radiation by butyl methacrylate at the wavelength required to excite the photoinitiator HMPP. Consequently other initiators with different excitation wavelengths were investigated as were photoinitiators that were likely to give higher rates of polymerisation. The structures and absorption spectra of these photoinitiators are shown in Figure 5-2 below.



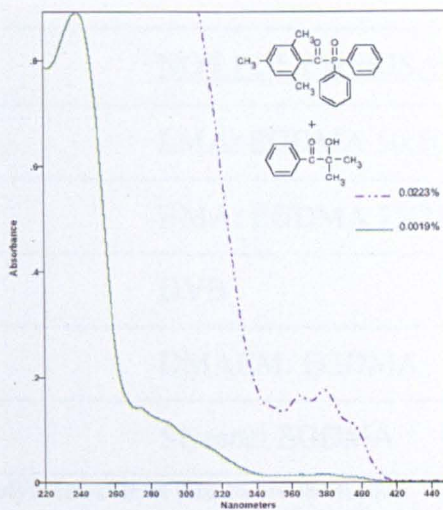
2-Methyl-4'-(methylthio)-2-morpholinopropiophenone (MMP)



2,2-dimethoxy-2-phenylacetophenone (DPP)



2-chlorothioxanthen-9-one (CTX)



Diphenyl(2,4,6-trimethylbenzoyl) phosphine oxide / 2-Hydroxy-2-methylpropiophenone, 50/50 blend (DPO / HMPP)

Figure 5-2 Structures and UV spectra of the photoinitiators 2-methyl-4'-(methylthio)-2-morpholinopropiophenone (MMP), 2,2-dimethoxy-2-phenylacetophenone (DPP), 2-chlorothioxanthen-9-one (CTX) and a 50:50 blend of diphenyl(2,4,6-trimethylbenzoyl) phosphine oxide (DPO) and 2-hydroxy-2-methylpropiophenone (HMPP). Spectral data reproduced from Aldrich Polymer Products Application and Reference Information[260].

The polymerisation of BMA with EGDMA with 1 wt % of each of the initiators above in glass vials after purging in a box a quartz window was investigated. All of these photoinitatiors were unable to achieve the polymerisation of butyl methacrylate. A range of other monomers were investigated to determine whether the polymerisation was unsuccessful due to characteristics peculiar to BMA or a common feature of the system being utilised. These monomers were lauryl methacrylate (LMA), hydroxyethylmethacrylate (HEMA), dimethylaminoethylmethacrylate (DMAEM), divinyl benzene (DVB) and styrene. The table below shows the compositions that were attempted; all compositions were tested with each initiator with concentrations of 1 wt % and 10 wt % and with methanol and DMSO as solvents and no solvent.

POLYMERISABLE	NOT POLYMERISABLE
EGDMA	LMA: EGDMA 50:50
BMA:EGDMA 50:50	BMA: EGDMA 75:25
HEMA:EGDMA any ratios	DVB
GMMA:EGDMA any ratios	DMAEM: EGDMA
GMMA:DVB 97.5: 2.5	Styrene: EGDMA

Table 5-1 Summary of monomer blends polymerisable in situ by uv intitiation

It appears that only strongly hydrophilic monomers are polymerisable by this method. It is believed that this is due to residual amounts of oxygen present in the system that acts as a an inhibitor that is only overcome where the monomer polymerises at a high rate. Consequently this technique is not suitable to the production of a polymer library. The lack of polymerisation in the other monomers is believed to be due to the relative depth of the monomer solution and it was decided to try the synthesis of thin films onto a surface.

5.1.2.3 Thin Films

Use of photoinitiators is an extremely well documented technique for the formation of thin films and coatings on a variety of substances. The requirements of a base material on which to coat the polymers for this application are as follows: optically transparent for visualisation of cells, inert to prevent interaction with assays, insoluble in organic and aqueous solvents, obtainable in a format whereby cells can be cultured on the surface and capable of retaining the coating. Candidates that meet these requirements include glass and PET sheets, previously used as disposable sheets laminated onto glass in the moulds. PET has the advantage that a coating can be applied to a large area and later cut to fit the container utilised for cell culture however it cannot be used with strong solvents such as TFA. The coatings formed by this technique should have the same characteristics as a sheet of polymer hydrogel as the random cross-linked structure can be formed without packing effect such as those formed with self-assembling monolayer and the film should be thick enough to prevent the base material from showing through.

Monomer solutions containing either GMMA or BMA were placed on a PET sheet and the liquid was drawn down the sheet using a 60μm coating bar. The sheet was then placed in the purging box where they were repeatedly evacuated and purged with nitrogen. All solutions were exposed to UV radiation for 5 minutes to ensure the same conditions were experienced by all the polymers. The coatings formed by this technique should have the same characteristics as a sheet of polymer hydrogel as the random cross-linked structure can be formed without packing effect such as those formed with self-assembling monolayer and the film should be thick enough to prevent the base material from showing through. After polymerisation the surfaces were washed with ethanol and then analysed to show that the polymerisation had been successful and that the polymer had been retained on the PET surface. The analysis of the surface was done by roughly ascertaining the surface free energy using a Dyne pen test set. The surface energy of uncoated PET was found to be 38-39 Dynes per cm^2 and any other surface energy was considered to have been coated successfully.

It was found that methanol, ethanol and isopropyl alcohol were all suitable solvents for coating as they were miscible with both glycerol methacrylate and butyl methacrylate and the mixtures wetted the PET surface. All the initiators trialled for the formation of polymers in situ were utilised to make thin films of glycerol methacrylate and butyl methacrylate and all were successful except for 2-chlorothioxanthen-9-one. Cell culture was performed on the thin films formed with different initiators to investigate the effect of the initiator on the cell adhesion and viability.

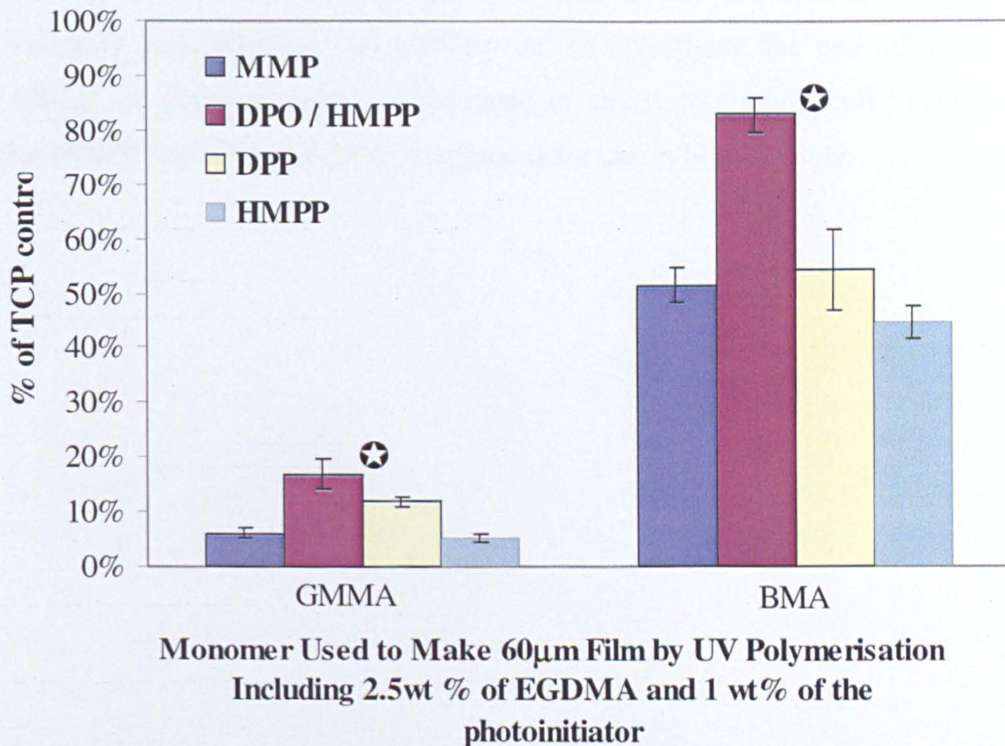


Figure 5-3 Human dermal fibroblast adhesion and survival on UV-initiated 60μm thin film random copolymers of GMMA or BMA containing 2.5wt % EGDMA and 1 wt % of the relevant initiator. 2-methyl-4'-(methylthio)-2-morpholinopropiophenone (MMP), 2,2-dimethoxy-2-phenylacetophenone (DPP), 2-hydroxy-2-methylpropiophenone (HMPP) or a 50:50 blend of diphenyl(2,4,6-trimethylbenzoyl) phosphine oxide (DPO) and HMPP. Cells (F1833, passage 9) were cultured on the substrates for 24 hours in media containing 10% FCS and were assessed by the MTT assay. Results shown are the mean of 3-6 replicates with standard errors shown. Students non-paired T-test was performed on the data, all polymers were significantly different to TCP ($p<0.01$) and ✪ indicates that $p<0.01$ compared to the other polymers of the same type synthesised with different initiators.

The polymers synthesised with the initiator DPO / HMPP were significantly better at supporting cell adhesion and viability than the polymers with the same monomer synthesised with any of the other initiators. All polymers supported approximately the same levels of cell adhesion and viability compared to tissue culture plastic as the thick sheets of polymers synthesised with thermal polymerisation through use of AIBN. Despite the greater ability of polymers made with DPO / HMPP to support cell adhesion and viability it was decided that HMPP alone was a better candidate for the synthesis of polymer libraries. The reasons for this are that the main aim of this research is not promote maximum cell adhesion and viability but to investigate the cell adhesion and viability on polymers with a wide range of ability to support cell function and that HMPP has FDA and MHRA approval for use in biomaterials.

5.1.3 Summary of Advantages & Disadvantages

Polymerisation Method	Advantages	Disadvantages
Thermal Curing	Any composition possible Well-defined technique Large amounts of peptide make concentration determinable by elemental analysis	Large amounts of polymer and peptide required Cracking caused by swelling and shrinkage Some polymers are opaque Inaccurate cell analysis
UV Curing: Sheets	Small amounts of polymer and peptide required	Limited choice of composition Likely to give inaccurate cell analysis
UV Curing: In Situ	Small amounts of polymer and peptide required Accurate cell analysis	Limited choice of composition Non-uniform surface Extensive washing required
UV Curing: Thin Films	Many compositions possible (limited by wettability of substrate on which coating is formed) Very small amounts of polymer and peptide required Accurate cell analysis	Difficult to verify presence of coating Analysis of RGD concentration impossible.

Table 5-2 The advantages and disadvantages of different polymerisation techniques.

The only two options available from the list above are the thermal polymerisation method and the thin-film method as the others do not fulfil the criteria that materials should be made by the same method and materials that do adsorb proteins should be incorporated into the polymer library.

The use of small amounts of peptide is not a requirement imposed by the design criteria but the basis of the synthesis of a library is that a large number of polymers can be included in the library. Whilst the need to minimise peptide usage was not a design criteria the thermal polymerisation techniques require 5g of monomers (and associated weight % of peptide) to provide 1 set of disks for

cell culture whereas the thin-film uv-initiated techniques requires just 0.2g of monomers to create 20 sets of disks for cell culture. The cost of the peptide required to create a library of polymers by the thermal polymerisation technique would be exorbitant. It was therefore decided to use the thin-film technique despite the inability to analyse the RGD concentration by amino acid analysis or elemental analysis.

5.2 Peptide Incorporation Techniques

RGD sequences containing a vinyl group can be easily synthesised by the addition of a species containing a vinyl group and a carboxylic acid group, such as methacrylic acid, at the end of the peptide chain in a manner similar to that used to form the chain. However the vinyl-containing peptide cannot be copolymerised directly with the other monomers as side chains on the peptide can react with the monomers. This was shown in section 3.4.1.3 where the same concentration of peptide was incorporation by copolymerisation and modification methods. When copolymerised the peptide promoted less than half the cell response obtained from the same concentration of peptide when immobilised by modification. Consequently protecting groups on the side chains of the peptide must be utilised to prevent loss of peptide activity.

5.2.1 Strategy 1: TFA Deprotection of Side Chains

Standard deprotections of side chains commonly utilised in peptide synthesis involves the addition of trifluoroacetic acid (TFA) in concentrations of 90-100%. This strong acid may affect the methacrylate groups by hydrolysing the esters and altering the properties of the polymer. Consequently the effect of the TFA on the polymers must be investigated and minimised.

Glycerol methacrylate hydrogels have been synthesised using the acetonide protected version of glycerol methacrylate (2-methyl-acrylic acid 2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester, GMAc) that was later deprotected with selenium dioxide and hydrogen peroxide[261]. The acetonide groups should be acid labile and could be removed at the same time as peptide protecting groups with 90-100% TFA. The mechanism for this reaction is shown overleaf in Figure 5-4.

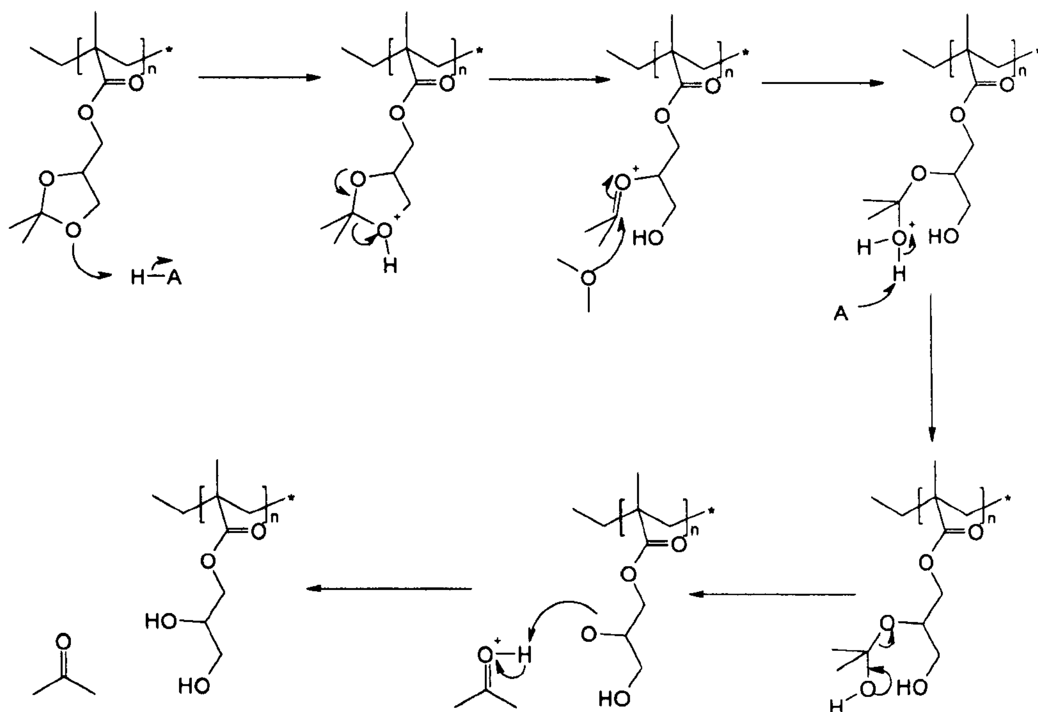


Figure 5-4 Mechanism for acid catalysed removal of acetonide protecting groups on poly(glycerol methacrylate.

It is also possible that the methacrylate ester could also be hydrolysed by the strongly acidic conditions and so the extent of deprotection and the concentration of acid groups in the final product were analysed.

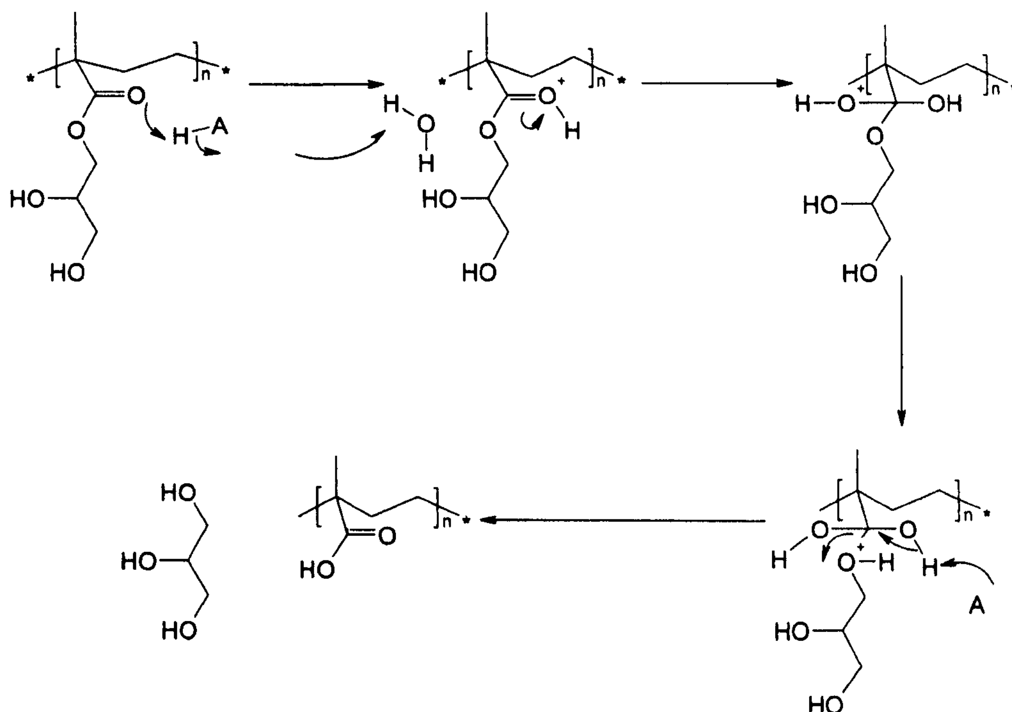


Figure 5-5 Acid catalysed hydrolysis of poly(glycerol methacrylate)

GMAc was synthesised by the reaction of solketal with methacryloyl chloride as shown in Figure 5-6 below.

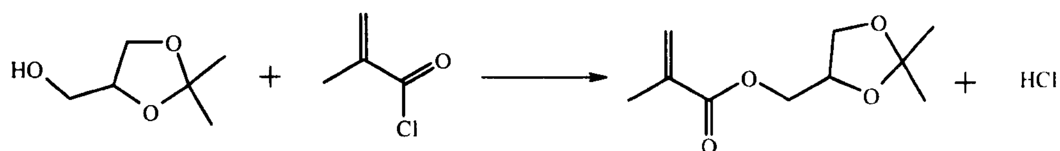


Figure 5-6 Synthesis of GMAc by reaction of solketal with methacryloyl chloride.

and a linear polymer was formed by free-radical polymerisation using the thermal initiator AIBN. The polymer was then purified, dried and incubated with neat TFA for 1 or 4 hours after which time the polymer was re-precipitated and analysed. The extent of the deprotection was analysed by ^{1}H NMR.

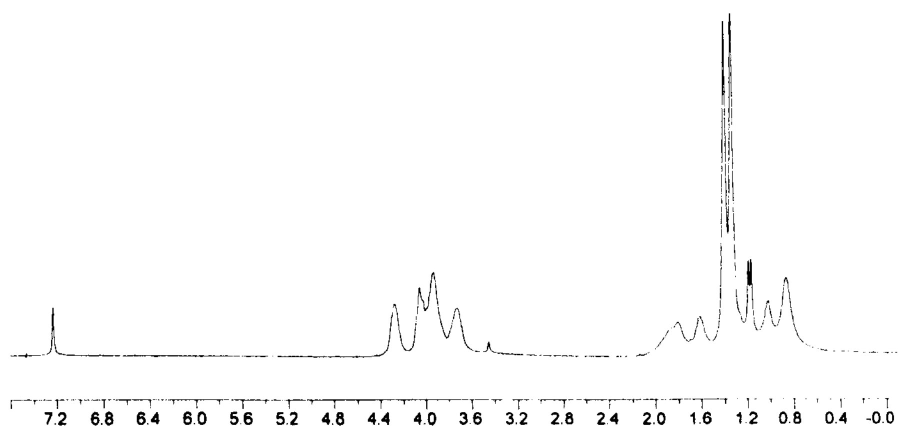


Figure 5-7 ^{1}H NMR of poly(GMAc)

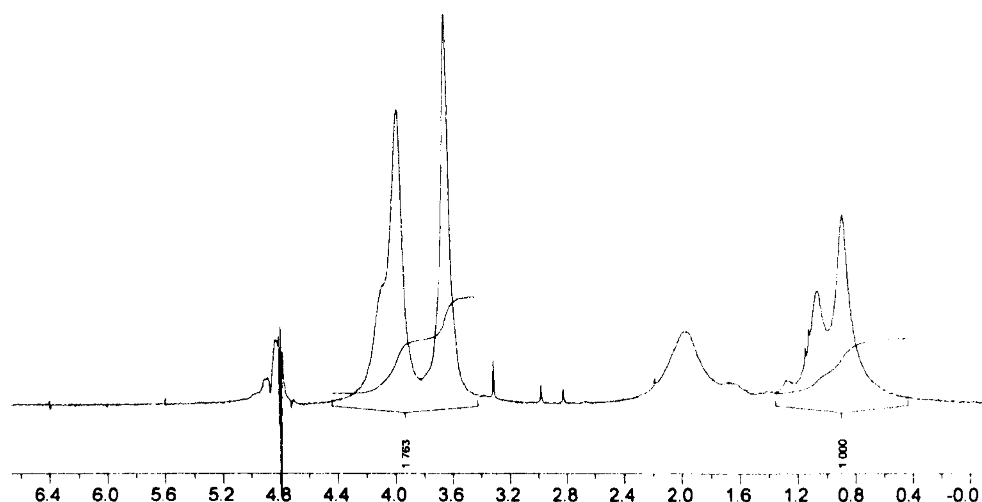


Figure 5-8 ^{1}H NMR of product of 4 hour reaction between poly(GMAc) and TFA

As shown by the NMR spectra in Figure 5-7 and Figure 5-8 above the peaks corresponding to the acetonide protecting groups at 1.2-1.5ppm were fully removed by the treatment with TFA. The extent of the acid catalysed ester

hydrolysis was analysed by titration of the treated polymer with aqueous sodium hydroxide. The average of three titrations gave a value of 6.6×10^{-6} moles of acid per gram of polymer. It was assumed that the entire mass of the polymer comprised of GMMA (there is a small percentage of the mass made up from initiator but this is assumed to be negligible) and this corresponds to 0.00625 molar equivalents of side chain functional groups per gram of polymer. This assumption in the calculation gave an average percentage of side chains that had been converted to carboxylic acid as being 0.105%. As the percentage of acid groups formed by the hydrolysis of the ester is extremely low it is assumed at this stage that this will not affect the physical or cell-culturing properties of the hydrogel.

Whilst the monomer GMAc can be utilised with TFA deprotection it is known that the cross-linker EGDMA is not stable towards acidic conditions. Hydrogels made with this cross-linker lose their integrity when exposed to strong acid and become difficult to handle in cell-culturing situations. An alternative cross-linker that is not degraded is divinyl benzene (DVB).

5.2.2 Strategy 2: Enzyme Deprotection

An ideal solution would be to find an alternative protection/deprotection method for the side chains of the peptide especially the arginine residue. The standard protecting groups on an arginine side chain are normally sulphonamides as these are stable to the reaction conditions required for peptide synthesis, prevent racemisation and are cleaved by the same conditions as cleavage of the peptide from the solid phase resin. A sulphonamide that is deprotected by weaker conditions than TFA in the hydrogel but is stable to the peptide synthesis conditions would fit the requirements of the synthesis however both strong acid and strong base are used in the peptide synthesis. Enzymatic deprotection of the sulphonamide group would fulfil all the criteria for the synthesis route if the sulphonamide was stable to the peptide synthesis conditions as enzymatic reactions generally take place in aqueous media, at neutral pH's and at moderate temperatures.

In 1999 Koeplinger et al and Zhao et al reported that the enzyme Glutathione-S-Transferase could deprotect benzyl sulphonamides[262]. Whilst this research did not show that arginine protecting groups could be removed it did

show that the deprotection was not dependant on the amine substitution pattern. The substituents on the amine side could affect the rate but the main factor in determining whether the deprotection would succeed was the substituents on the benzene ring. Electron-withdrawing substituents ortho / para to the sulphonamide allowed the deprotection to be accomplished. Some of the compounds that were investigated by Zhao et al and indicate the structure-activity relationship of this reaction are shown below.

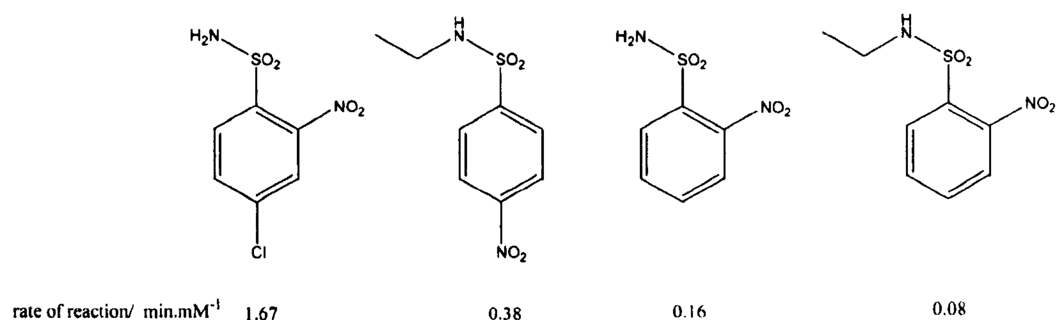


Figure 5-9 Structure-Activity relationship of the deprotection of benzylsulphonamides by Glutathione-S-Transferase as reported by Zhao et al[262].

The rate of sulphonamide deprotection is increased when the electron-withdrawing substituent is para to the sulphonamide and decreased when the amine is more substituted. Consequently we investigated the potential for this deprotection strategy to be utilised with arginine residues as an alternative for the construction of a hydrogel library.

Enzymatically cleavable protecting groups have been investigated previously in peptide synthesis to provide orthogonal protecting strategies that compliment the existing chemistries available to peptide chemists. These techniques have not achieved wide-spread usage as there is insufficient demand for additional methods of peptide synthesis. However these show that the targeted use of enzymes to deprotect peptide sequences should be successful in theory if the protecting group and enzyme are carefully selected.

Enzymatically cleavable protecting groups have been utilised to synthesise model nucleoproteins where a side chain OH from serine in a short peptide sequence was linked via a phosphodiester group to a nucleic acid[263]. Due to the number of different functional groups present in nucleopeptides current techniques of orthogonal protecting strategies could not be used to synthesise the nucleopeptides. A phenylacetoxy benzyloxycarbonyl (PhAcOz) group (urethane) was utilised to protect the amine terminus of a peptide containing serine prior to

formation of the phosphodiester bond and was later removed by the enzyme Penicillin G Acylase. The enzyme catalysed hydrolysis occurred at RT in PBS (pH 6.8) containing 20% methanol and the desired product was isolated with yields of 60-69% dependent on the flanking amino acid residues.

A glucose based enzymatically cleavable group tetrabenzylglucosyloxycarbonyl (BGloc) has been used as a protecting group for the amine terminus of peptide sequences[264]. The removal of the protecting group was achieved in two steps by the hydrogenation of the glucose benzyl ester followed the removal of the glucose group by α and β glucosidase. Yields of 67-91% were obtained from this two-step procedure.

Phenyl hydrazide groups have been used to protect the carboxyl terminus of peptides and can be removed by tyrosinase[265]. Yields obtained from the enzymatic deprotection were in the order of 64-99% depending on the amino acid and / or length of the peptide sequence.

Although no protection and enzymatic cleavage of arginine side chains has been conducted this research shows that enzymes can be utilised to cleave protecting groups on peptides in high yields under mild conditions.

5.3 Amino Acid Synthesis and Deprotection

The work of Zhao et al showed that virtually any benzyl sulphonamide could be cleaved by GST enzymes if there were electron withdrawing substituents on the benzene ring ortho or para to the sulphonamide. In order to synthesise a peptide containing an arginine protected with the benzyl sulphonamide the amino acid must be synthesised with Fmoc protection as the peptide will be synthesised on a solid support using Fmoc chemistry[266,267]. In addition the benzyl sulphonamide must be stable to strong acid (TFA for cleavage) and strong base (20% piperidine for removal of Fmoc groups).

The synthesis route to forming protected arginine derivatives usually involves the reaction of Z-Arginine with a sulphonyl chloride. The Z group can then be removed by hydrogenation and the Fmoc group added by reaction with Fmoc-Cl. As this route requires the use of hydrogenation to remove the Z group then $-NO_2$ cannot be used as the electron-withdrawing substituent as it would be converted to $-NH_2$. A survey of commercially available benzylsulphonyl

chlorides identified 4-bromobenzyl sulphonyl chloride as a target. This reaction scheme is shown in Figure 5-10 below.

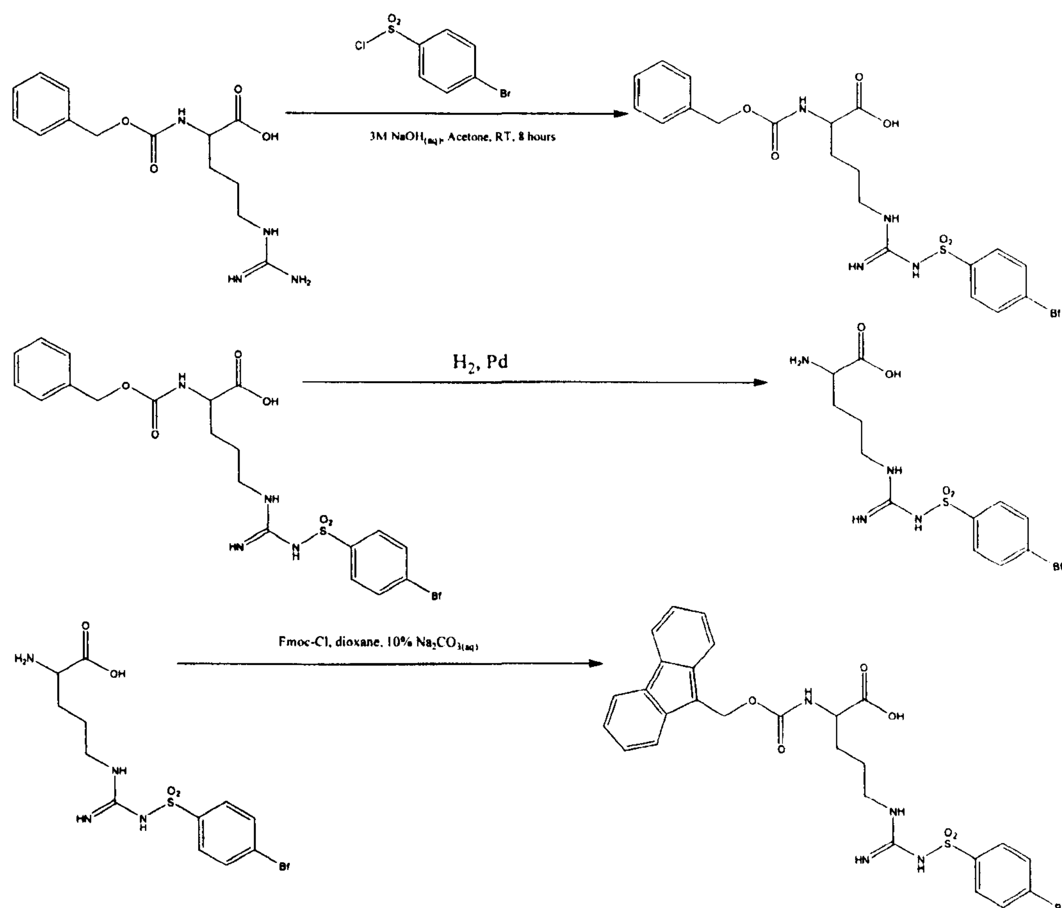


Figure 5-10 Synthesis of Fmoc-Arg(4-Bbs) from Z-Arg-OH

5.3.1 Synthesis of Z-Arg(4-Bbs)

Z-Arg(4-Bbs) was successfully synthesised by the reaction of Z-Arg-OH with 4-bromobenzylsulphonyl chloride in acetone and 3M sodium hydroxide solution as shown in Figure 5-11.

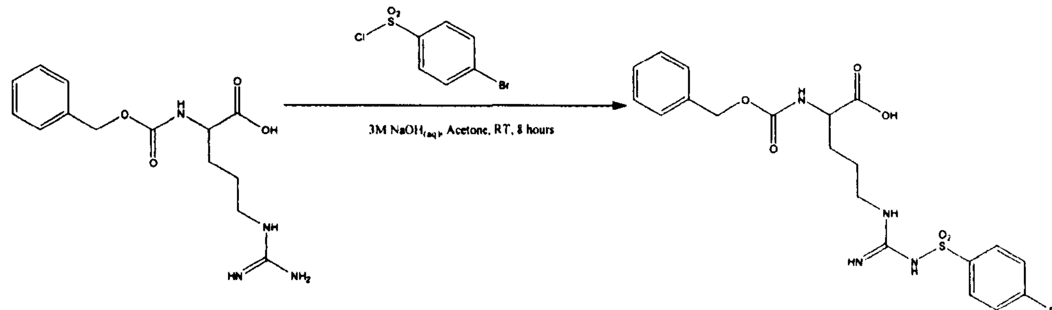


Figure 5-11 Reaction of Z-Arg-OH with 4-bromonbenzyl sulphonyl chloride.

This method was developed as an adaptation of the procedures used to synthesize arginine derivatives with different sulphonyl protecting groups[268]. 20ml of $NaOH_{(aq)}$ 3M per g of Z-Arg-OH and 1.1equivalents of the sulphonyl chloride were used. The product was found to be a cream-coloured gum that

could be converted into a solid by adding cyclohexylamine (CHA) to form the salt. The yield of the reaction was found to be low, approximately 20% and the purity of the product (analysed by HPLC with diode array and mass spectrometry detection) was also poor. The removal of the CHA by solvent extraction with dilute sulphuric acid further reduced the yield of the product to an overall 10%. The formation of the CHA was abandoned as this yield was deemed to be unacceptable. It was found that the product could also be formed as a solid by not drying the solvents and precipitating into ether. It is believed that the product formed is hydrated.

Yields were improved to approximately 50% by utilising 3 eq. of the sulphonyl chloride and by utilizing dioxane as the solvent. Attempts to recrystallize the product from various solvents (water, ethyl acetate, methanol, acetonitrile and combinations thereof) failed, which is not surprising, as the natural state of the product appears to be a gum and not a solid. Purification of the product could be achieved by repeated precipitation into diethyl ether from methanol or ethyl acetate but the yields were reduced by this process to less than 10%. Extraction of the product into 10% sodium carbonate solution, acidification of the solution and then re-extraction back into ethyl acetate had more success. Final reaction yields were in the order of 40% and the purity was greater than 85% by HPLC. This level of purity was deemed insufficient for testing the deprotection efficiency of the enzyme and so the product was further purified by preparative HPLC.

5.3.2 Determination of Deprotection by Sulphite Assay

The reaction of GST with a benzyl sulphonamide produces a benzyl group co-ordinated to GSH, an amine and sulphur dioxide. Sulphur dioxide dissolves in water to form sulfite and this was utilised by Zhao et. al. to determine the rates of reaction of GST with various benzyl sulphonamides. The determination of the sulfite concentration was achieved by the reaction of Basic Fuchsin (otherwise known as pararosaniline HCl) with the sulfite and the spectrophotometric analysis at 590 nm. GSH was known to interfere with the spectrophotometry and consequently was precipitated out with mercury II chloride before the analysis was performed.

Stock solutions of sodium sulfite containing 500 μM GSH were prepared and utilised to form a calibration chart.

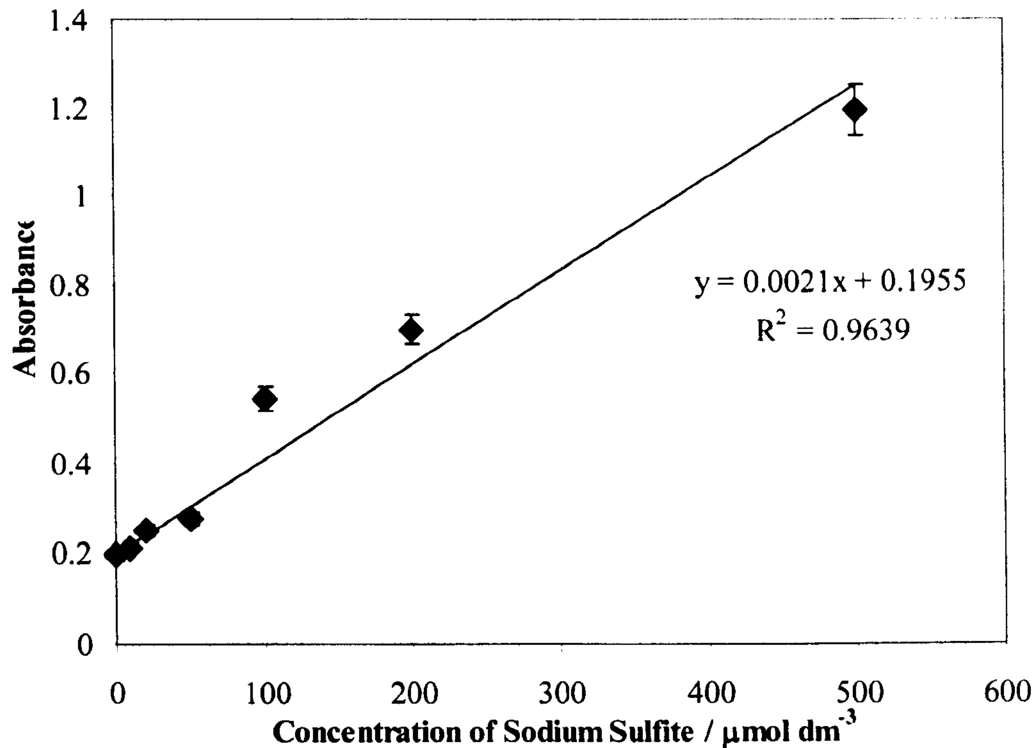


Figure 5-12 Calibration chart showing the concentration of sulfite as determined by the basic Fuchsin colorimetric assay. Results shown are the average of 3 replicates with standard deviations shown.

The calibration chart in Figure 5-12 gave a linear fit and showed that the sulfite concentration could be accurately determined up to 50μM concentration and approximately determined from 100 to 500μM. It is believed that the loss in accuracy at higher concentrations is the result of the adsorption of the sulfite onto the GSH precipitate. The concentration of sulfite formed from the reaction of varying concentrations of amino acid with varying concentrations of GST were examined at 24 and 48 hour time points.

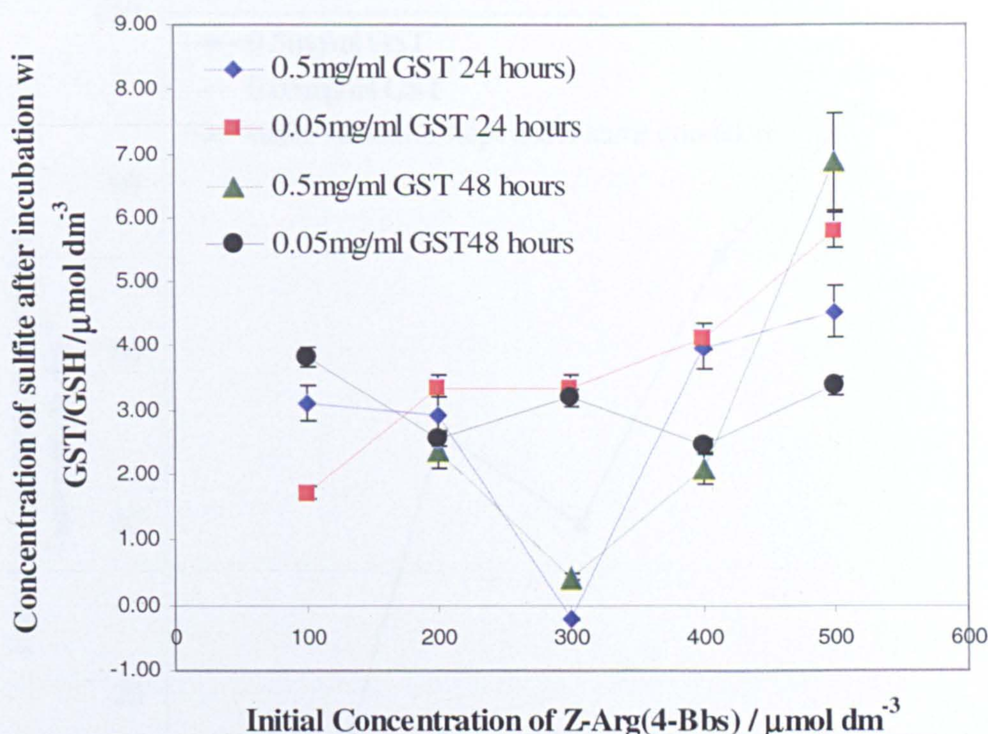


Figure 5-13 Deprotection of Z-Arg(4-Bbs) analysed via sulfite Formation and basic fuchsin Colorimetric Assay. Results are the mean of 3 replicates with standard deviations shown.

The results of this experiment shown in Figure 5-13 were disappointing as, although the results clearly showed some deprotection the amount of sulfite present was very low. The maximum sulfite concentration observed was 7.5 µM, only 1.5% of the total concentration of the amino acid.

It was believed that the calibration chart may be inaccurate due to the loss of sulfite as the gas sulphur dioxide and the experiment was repeated with the sodium sulfite standards being kept in the same conditions as the enzyme reactions.

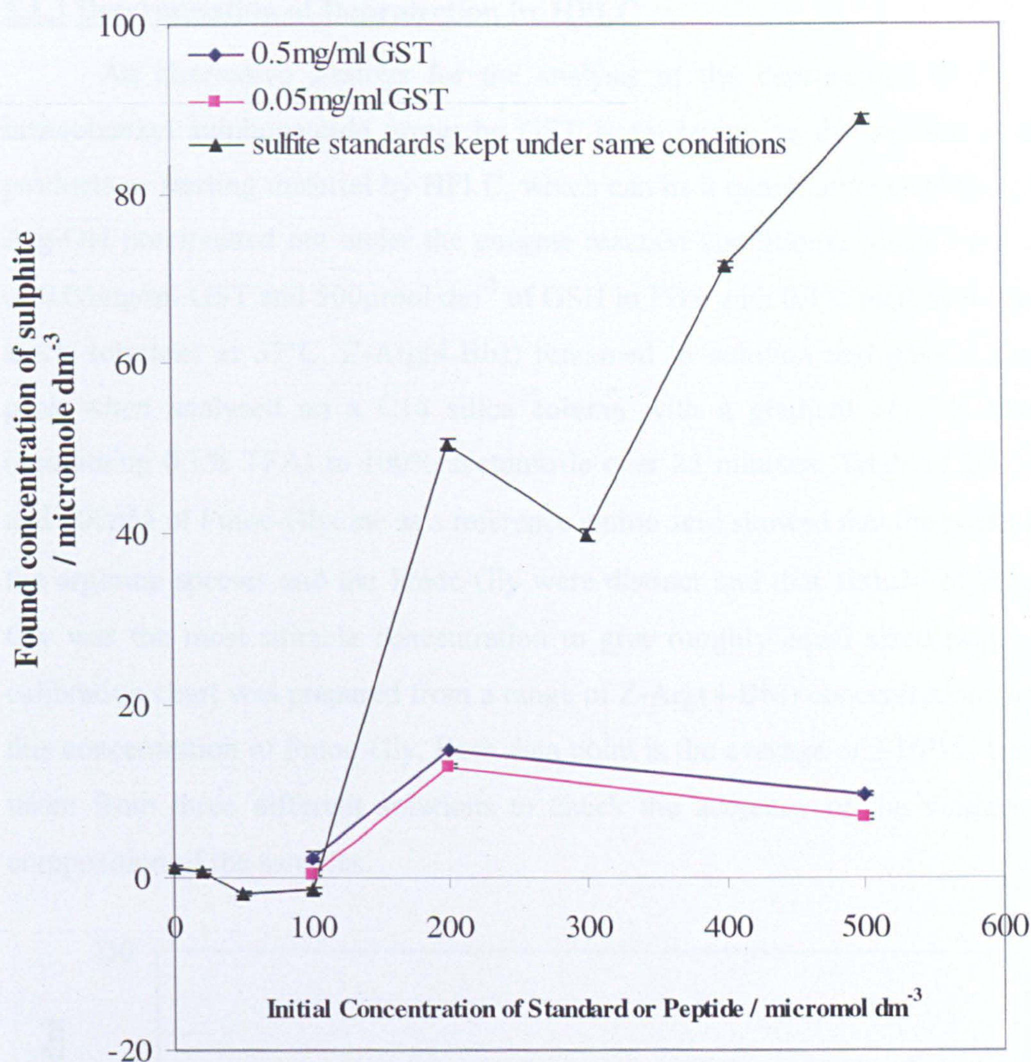


Figure 5-14 Graph showing sulfite concentration generated by deprotection of Z-Arg(4-Bbs) and remaining sulfite concentration in sulfite standards kept under the same conditions.

In Figure 5-14 the sodium sulfite standards no longer gave a linear plot after incubation in the same conditions that the amino acid deprotection solutions were subjected to and so it was impossible to determine the concentration of sulfite formed in the enzyme reaction from the absorbance. In addition, when the absorbances from the sulfite standards are applied to the original calibration chart, it appears that significant amounts of the sulfite are lost as sulphur dioxide. This indicates that the sulfite concentrations determined in the first experiment are a combination of the amount of sulfite formed and the amount of sulphur dioxide lost to the atmosphere, and as such are more indicative of the rate of reaction rather than the total amount of amino acid deprotected.

5.3.3 Determination of Deprotection by HPLC

An alternative strategy for the analysis of the deprotection of the 4-bromobenzyl sulphonamide group by GST is to determine the amount of the products or starting material by HPLC, which can be a quantitative technique. Z-Arg-OH precipitated out under the enzyme reaction conditions, which were 0.5 or 0.05mg/ml GST and 500μmol dm^{-3} of GSH in PBS with 0.1% methanol (from stock solution) at 37°C. Z-Arg(4-Bbs) remained in solution and gave a sharp peak when analysed on a C18 silica column with a gradient of 95% water (containing 0.1% TFA) to 100% acetonitrile over 25 minutes. Trials of 20, 100 and 500μM of Fmoc-Glycine as a reference amino acid showed that the peaks for the arginine species and the Fmoc-Gly were distinct and that 100μM of Fmoc-Gly was the most suitable concentration to give roughly equal sized peaks. A calibration chart was prepared from a range of Z-Arg(4-Bbs) concentrations with this concentration of Fmoc-Gly. Each data point is the average of 3 HPLC traces taken from three different solutions to check the accuracy of the volumetric composition of the samples.

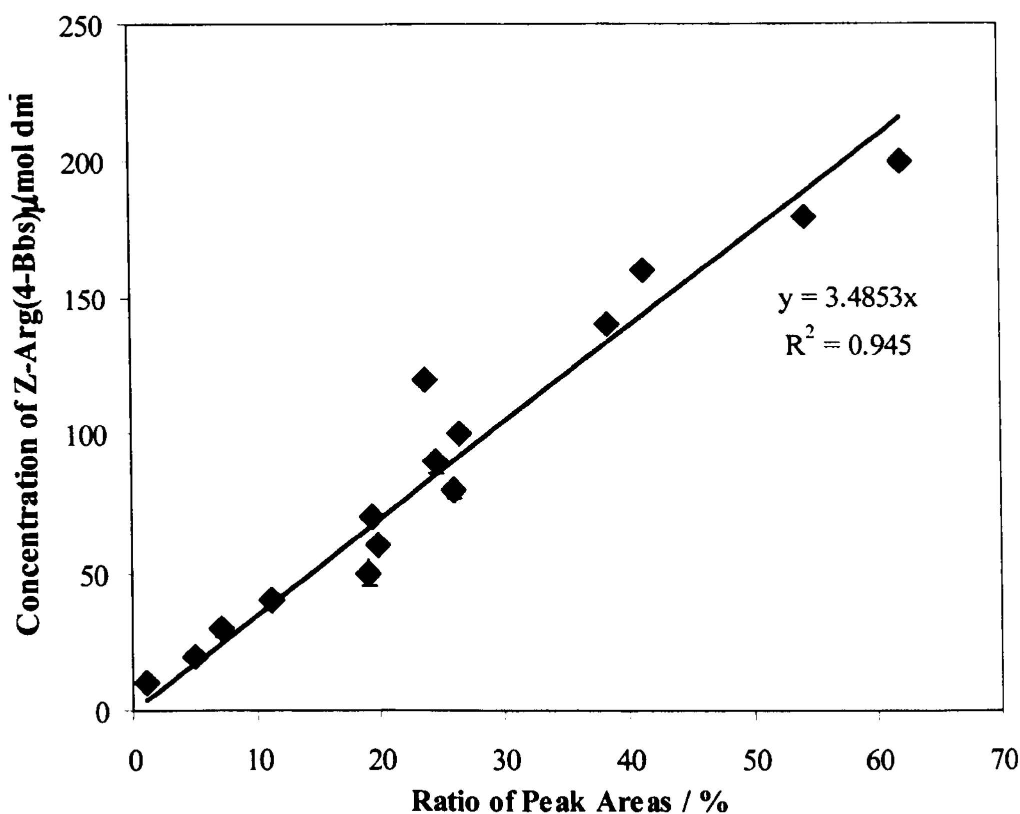


Figure 5-15 Calibration chart showing the concentration of Z-Arg(4-Bbs) as determined by the relative peak area compared to Fmoc-Gly. Results shown are the mean of 5 replicates with standard errors shown.

The calibration plot in Figure 5-15 shows the expected linear relationship between the concentration of the protected arginine species and the ratio of the peak area compared to that of the reference amino acid. There are some errors introduced through inaccuracies in sample preparation or analysis method and these must be taken into account when assessing the reliability of the data.

Three different concentrations of Z-Arg(4-Bbs) were reacted with GST at two different concentrations at room temperature for 24 hours and then analysed by HPLC. In all cases, allowing for errors, there were no detectable reductions in the concentration of the arginine species and so the experiment was repeated with a reaction time of 7 days.

Conc. of Z-Arg(4-Bbs) used / µM	Conc. of GST / mg/ml	Ratio of peak areas (%)	Conc. of Z-Arg(4-Bbs) found / µM	Amount converted / µM	Percentage converted
50	0.5	9.93	34.46	15.54	31.08 %
100	0.5	17.81	61.81	38.19	38.19 %
200	0.5	33.91	117.67	82.33	41.17 %
50	0.05	10.37	35.99	14.01	28.02 %
100	0.05	21.30	73.92	26.08	26.08 %
200	0.05	49.13	170.50	29.50	14.75 %

Table 5-3 Deprotection of Z-Arg(4-Bbs) by incubation with 0.05 or 0.5mg/ml GST and 500 µmol dm^{-3} GSH for 7 days at room temperature as determined by HPLC.

In all cases the concentration of Z-Arg(4-Bbs) had decreased showing that the benzyl sulphonamide had been deprotected by the enzyme. The amount that had been deprotected appears to be proportional to some degree to the concentration of both the amino acid and the enzyme. It is possible to assume that these reactions have not reached equilibrium as the percentage conversion differs in each case. From this it is a reasonable assumption that the amount deprotected at time T is proportional to the rate of the reaction. As these results show a linear increase in amount deprotected at time T they imply that the rate of the reaction is first order with respect to both the amino acid and the enzyme (see Figure 5-16).

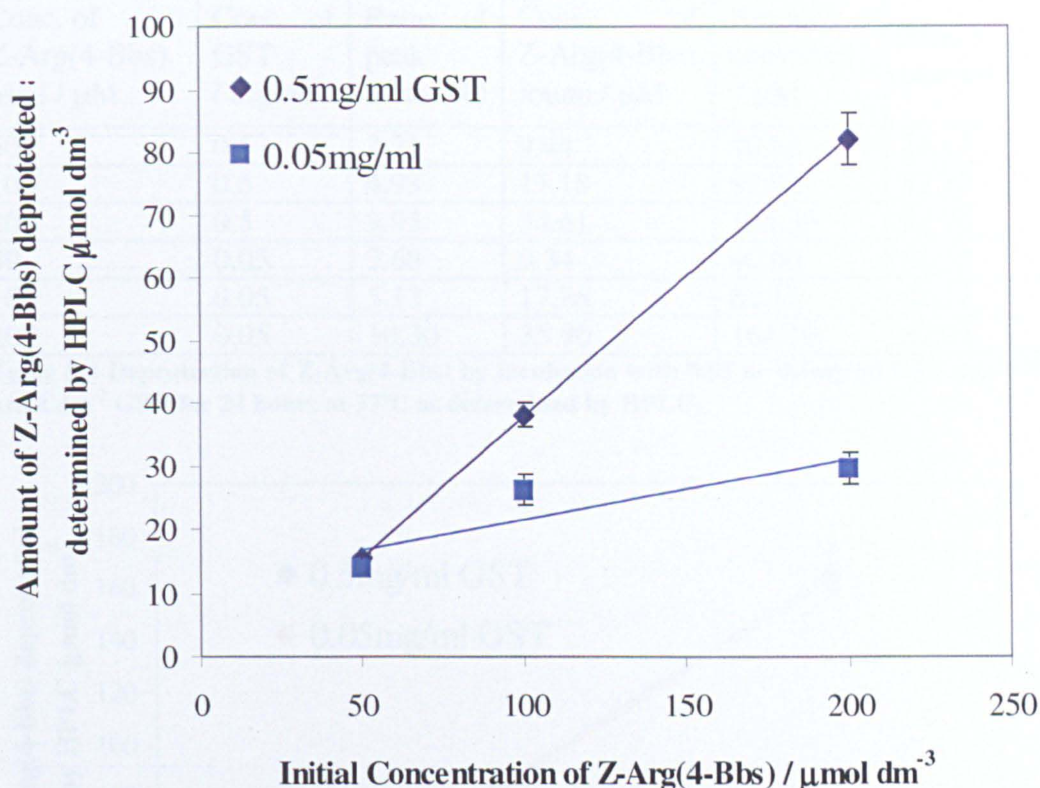


Figure 5-16 Deprotection of Z-Arg(4-Bbs) by incubation with 0.05 or 0.5mg/ml GST and 500 μmol dm^{-3} GSH for 7 days at RT as determined by HPLC with respect to the reference amino acid Fmoc-Glycine

Whilst these results show that the benzyl sulphonamide species can be deprotected by the enzyme the reaction time is too long and the percentage conversion is too low for this to be of use in a synthetic strategy. However the physiological conditions under which the enzyme normally functions involve a higher temperature than used in these experiments. These experiments were therefore repeated with the reaction solutions being incubated at 37°C. A new calibration chart was also prepared with the calibration solutions being kept under the same conditions as the reactions. The data from these experiments can be found in Table 5-4 and Figure 5-17 below.

Conc. of Z-Arg(4-Bbs) used / μM	Conc. of GST / mg/ml	Ratio of peak areas (%)	Conc. of Z-Arg(4-Bbs) found / μM	Amount converted / μM	Percentage converted
50	0.5	2.71	9.44	40.56	81.11
100	0.5	4.93	17.18	82.82	82.82
200	0.5	9.93	34.61	165.39	82.70
50	0.05	2.68	9.34	40.66	81.32
100	0.05	5.13	17.88	82.12	82.12
200	0.05	10.30	35.90	164.10	82.05

Table 5-4 Deprotection of Z-Arg(4-Bbs) by incubation with 0.05 or 0.5mg/ml GST and 500 μmol dm^{-3} GSH for 24 hours at 37°C as determined by HPLC.

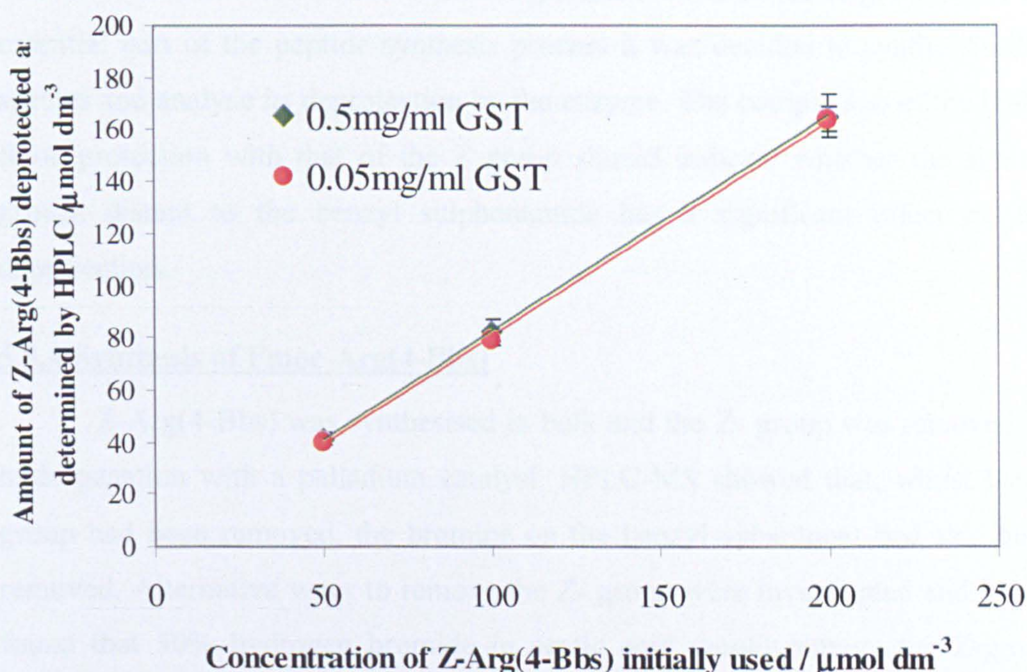


Figure 5-17 Deprotection of Z-Arg(4-Bbs) by incubation with 0.05 or 0.5mg/ml GST and 500 μmol dm^{-3} GSH for 24 hours at 37°C as determined by HPLC with respect to the reference amino acid Fmoc-Gly. Results shown are the mean of 3 replicates with standard deviations shown.

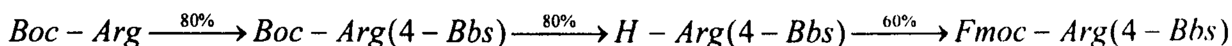
These results clearly show that the same level of deprotection is achieved with both concentrations of GST (0.5 and 0.05 mg/ml). In addition they also show that the reaction has reached equilibrium over a 24 hour period with a percentage conversion of ~82%. Both the time scale and the degree of deprotection indicate that this method would be suitable for the protection strategy in the peptide. In addition it is possible that the conversion in the

polymer would be driven to completion by the fact the peptide would be tethered to the support, removing one of the products from solution and preventing the reverse reaction. However the mobility of the solvated polymer chains in aqueous media means that the reaction of species attached to swollen hydrogels can behave more like species in a viscous solution. It is also possible that the incorporation of the arginine residue into a peptide sequence and immobilisation into a polymer could hamper the deprotection by steric hindrance of the enzyme's access to the protecting group. Consequently it was decided to analyse the effect of steric hindrance on the deprotection. As Fmoc-Arg(4-Bbs) is an essential part of the peptide synthesis process it was decided to synthesise this species and analyse its deprotection by the enzyme. The comparison of the bulky fmoc protection with that of the Z group should indicate whether the size of groups distant to the benzyl sulphonamide has a significant effect on the deprotection.

5.3.4 Synthesis of Fmoc-Arg(4-Bbs)

Z-Arg(4-Bbs) was synthesised in bulk and the Z- group was removed by hydrogenation with a palladium catalyst. HPLC-MS showed that, whilst the Z group had been removed, the bromine on the benzyl substituent had also been removed. Alternative ways to remove the Z- group were investigated and it was found that 30% hydrogen bromide in acetic acid would remove the Z-group whilst leaving the side chain protecting group intact. The stability of the protecting group towards strong acids and bases is one of the factors that makes it appealing as it can be used as an alternative orthogonal protection strategy for other uses as well as copolymerisation of peptides. The extremely corrosive and toxic nature of the HBr/AcOH method however made it unsuitable for use in a large scale synthesis of H-Arg(4-Bbs). It was decided to alter the original protecting group from Z to Boc as the Boc group is more easily removed by TFA. Boc-Arg(4-Bbs) was synthesised and it was found that this strategy had additional benefits as the yields were higher (70-90%) and the re-extraction process gave a higher degree of purity. The deprotection of Boc-Arg(4-Bbs) with TFA was achieved as expected as was the reaction of H-Arg(4-Bbs) with Fmoc-Cl[269] to form the final protected amino acid to be utilised in both enzyme

deprotection analysis and peptide synthesis. Typical overall yields were in the order of 40%.



$$Boc-Arg \xrightarrow{80\%} Boc-Arg(4-Bbs) \xrightarrow{80\%} H-Arg(4-Bbs) \xrightarrow{60\%} Fmoc-Arg(4-Bbs)$$

5.3.5 Deprotection of Fmoc-Arg(4-Bbs)

Fmoc-Arg(4-Bbs) of varying concentrations was incubated with 0.05mg/ml of GST for 24 hours. Calibration solutions containing varying concentrations Fmoc-Arg(4-Bbs), 500μM GSH and varying concentrations of Fmoc-Gly were also prepared and incubated in the same conditions. The extent of the deprotection was determined by the average peak areas of the amino acid remaining compared to that of the reference amino acid. The initial reference amino used was Fmoc-Gly as it was the same as that used in the analysis of Z-Arg(4-Bbs) deprotection. Trials with Fmoc-Gly showed that a concentration of 100μM was most suitable as it gave reasonable sized peaks relative to those of Fmoc-Arg(4-Bbs) at concentrations ranging from 20 to 200μM. However when solutions using Fmoc-Gly as a reference amino acid were incubated in PBS with 500μM GSH the average peak area of the Fmoc-Gly reduced drastically. It is unknown why this should be the case when the only change from previous experiments was to alter the protecting group on the arginine species. Consequently the reference amino acid was changed to Boc-Phe at a concentration of 1000μmol dm^{-3}.

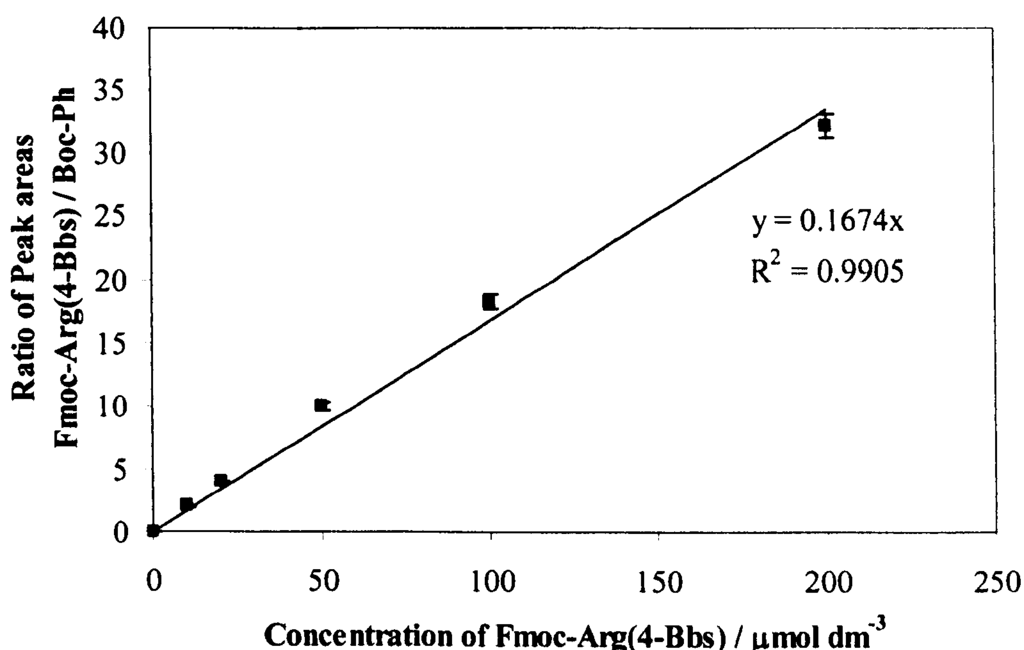


Figure 5-18 Calibration Chart showing the concentration of Fmoc-Arg(4-Bbs) as determined by the relative peak area compared to Boc-Phe. Results shown are the mean of 3 replicates with standard deviations shown.

With Boc-Phe as the reference amino acid the calibration chart was linear as shown in Figure 5-18 even after the standards have been incubated for 24 hours at 37°C (reaction conditions).

Conc. of Fmoc-Arg(4-Bbs) used / μM	Conc. of GST / mg/ml	Ratio of peak areas (%)	Conc. of Fmoc-Arg(4-Bbs) found / μM	Amount converted / μM	Percentage converted
50	0.5	7.16	38.29	11.70	23.41
100	0.5	14.40	77.03	22.97	22.97
200	0.5	28.77	153.92	46.08	23.04
50	0.05	7.34	39.27	10.73	21.45
100	0.05	14.47	77.42	22.58	22.58
200	0.05	28.86	154.40	45.6	22.80

Table 5-5 Deprotection of Fmoc-Arg(4-Bbs) by incubation with 0.05 or 0.5mg/ml GST and 500 μmol dm^{-3} GSH for 24 hours at 37°C as determined by HPLC

These results shown in table 5 indicate that deprotection has occurred and that, as with the Z protection, equilibrium has been reached after 24 hours incubation. However it is clear that the bulky fmoc group has had an adverse effect on the percentage deprotection of the side chain reducing it from ~82% to ~22%. This implies that, whilst achievable, the presence of the polymer surrounding the protecting group may also have an adverse effect on the percentage deprotection. Alternatively, as discussed earlier, the heterogenous nature of the reaction when the peptide is incorporated in the cross-linked polymer may move the equilibrium position as local regional effects are likely to have a large effect on the deprotection. It was therefore decided to proceed with the peptide and polymer library synthesis and analyse the deprotection *in situ*. In addition the peptide that has not been deprotected should serve as an adequate control sequence similar to a scrambled or substituted peptide that shows the effect of the peptide presence on non-specific (i.e. not integrin mediated) cell adhesion to a material.

5.4 Peptide Synthesis

It is known that the minimal peptide sequence RGD has a low affinity for integrins and slightly longer sequences such as RGDS, RGDY and RGDC have higher affinities. It has also been shown that in certain polymer systems a spacer arm is required to distance the peptide from the bulk of the polymer. However the conditions under which the spacer arm is required have not been elucidated and it was decided to investigate this requirement in these systems being investigated by synthesising peptides both with and without a spacer arm. In addition the additional distance between the peptide and the bulk of the polymer may reduce steric hindrance and allow better deprotection by the enzyme. It was also decided to use the sequence GRGDS as this sequence has been frequently utilised in previous publications and is known to promote cellular functions in polymeric materials. The target peptides are shown in Figure 5-19 (fully protected peptides) and Figure 5-20 (with arginine protected by 4-bromobenzylsulphonyl group).

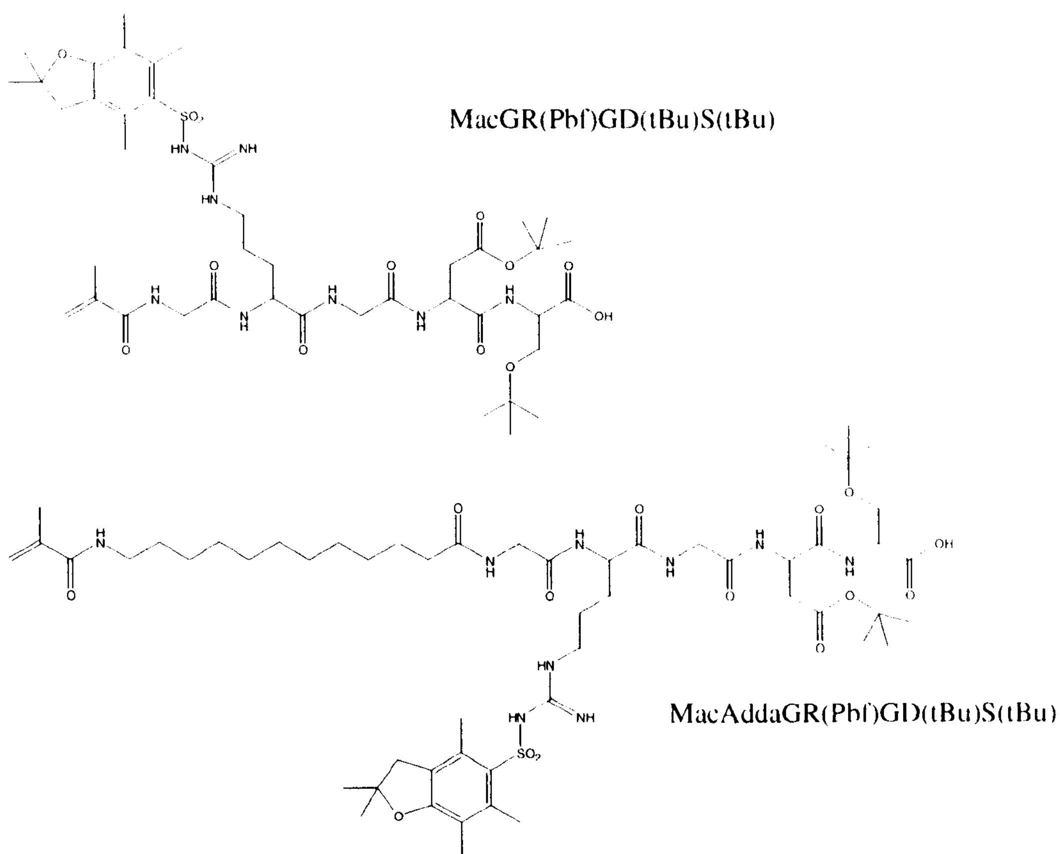


Figure 5-19 Target protected peptides for use with strategy 1: TFA deprotection (protecting groups shown in red)

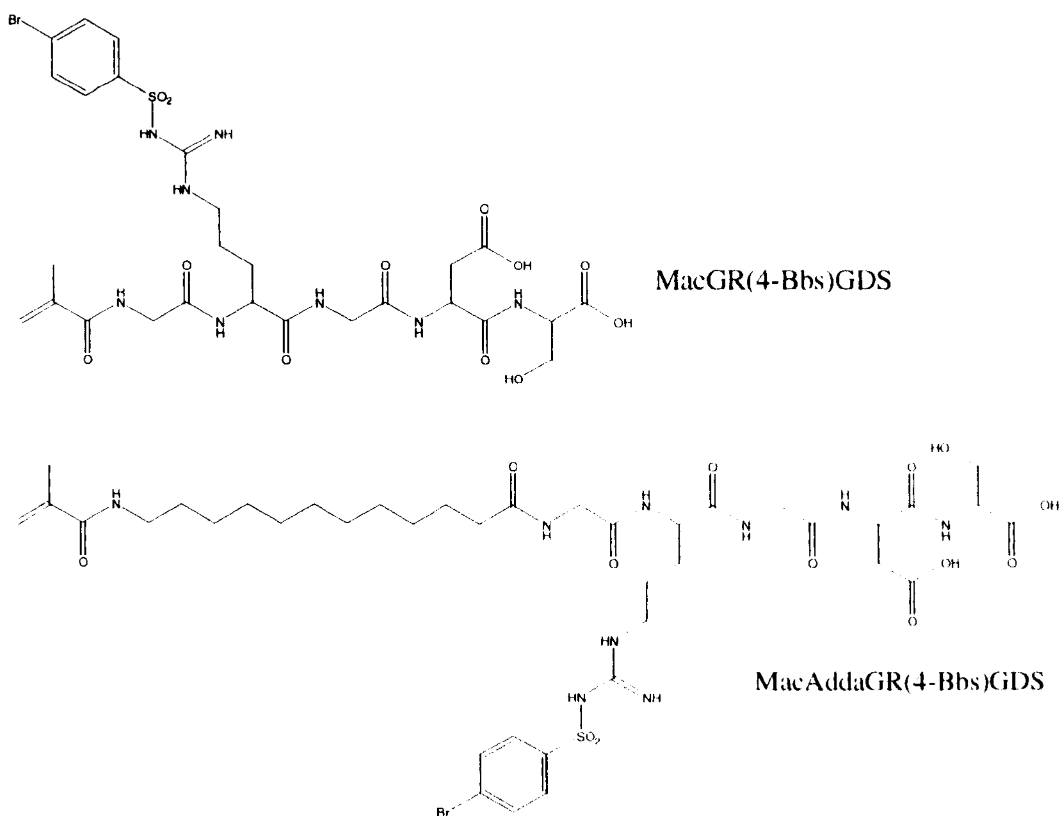


Figure 5-20 Target peptides for use with strategy 2: Enzyme deprotection (protecting groups shown in blue)

The first step of peptide synthesis is to attach the first amino acid to a solid support. The peptides with enzyme labile protecting groups are synthesised on Wang resins, which can be bought with the first amino acid pre-attached. The fully protected peptides are synthesised on Rink acid labile resins and the first amino acid must be attached to the resin by MSNT mediated reaction. This reaction is shown below in Figure 5-21.

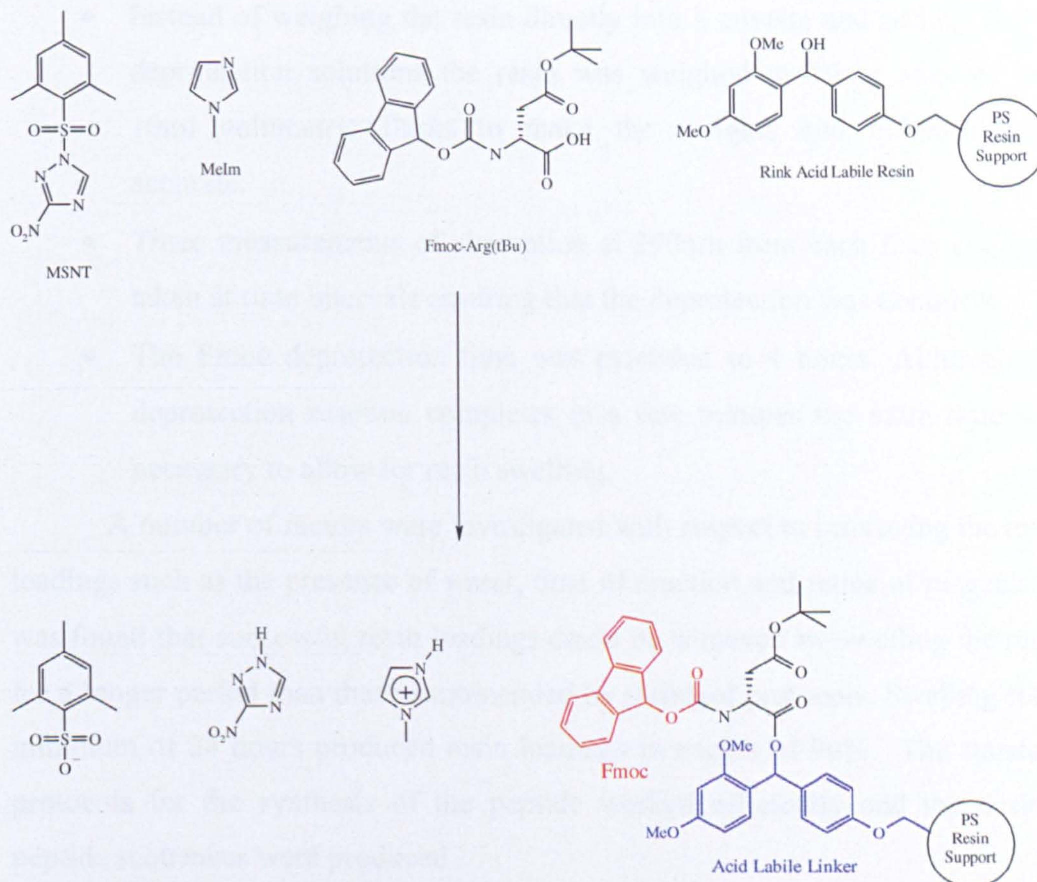


Figure 5-21 Reaction scheme for attachment of first amino acid to Rink acid labile resin by use of MSNT

The first attempts to attach an amino acid to a rink acid labile resin[270] using standard MSNT protocols[1266,271] were unsuccessful as they resulted in a

[1] 5eq of the amino acid, 5 eq. of MSNT, 3.75 eq of methyl imidazole and DCM 10ml per g of resin added to resin pre-swollen in DCM and purged with nitrogen for 1 hour, allowed to react for 1 hour with N_2 agitation then washed with 3 aliquots DCM and 3 aliquots methanol.

low resin loadings of 0.1-0.4 mmol /g. The nominal loading capacity of the resin is 0.6-0.8mmol / g making the functionalised resin unsuitable for further synthesis as this requires greater than 70% functionalisation. One of the problems appeared to be unsatisfactory errors in the assessment of the resin loadings as one sample gave results of 30% conversion and 60% conversion – clearly an unacceptable error. The protocol for the analysis of the resin loading was modified in the following ways:

- Instead of weighing the resin directly into a cuvette and adding 3ml of deprotection solutions the resin was weighed in 10mg aliquots into 10ml volumetric flasks to make the weights and volumes more accurate.
- Three measurements of absorption at 290nm from each flask could be taken at time intervals ensuring that the deprotection was complete.
- The Fmoc deprotection time was extended to 4 hours. Although the deprotection reaction completes in a few minutes the extra time was necessary to allow for resin swelling.

A number of factors were investigated with respect to improving the resin loadings such as the presence of water, time of reaction and ratios of reagents. It was found that successful resin loadings could be achieved by swelling the resin for a longer period than that recommended by standard protocols. Swelling for a minimum of 24 hours produced resin loadings in excess of 90%. The standard protocols for the synthesis of the peptide worked efficiently and the desired peptide sequences were produced.

The Fmoc protecting groups were removed by treatment with 20% piperidine in DMF for 3 minutes then 7 minutes. The mechanism of Fmoc removal is shown in Figure 5-22 overleaf.

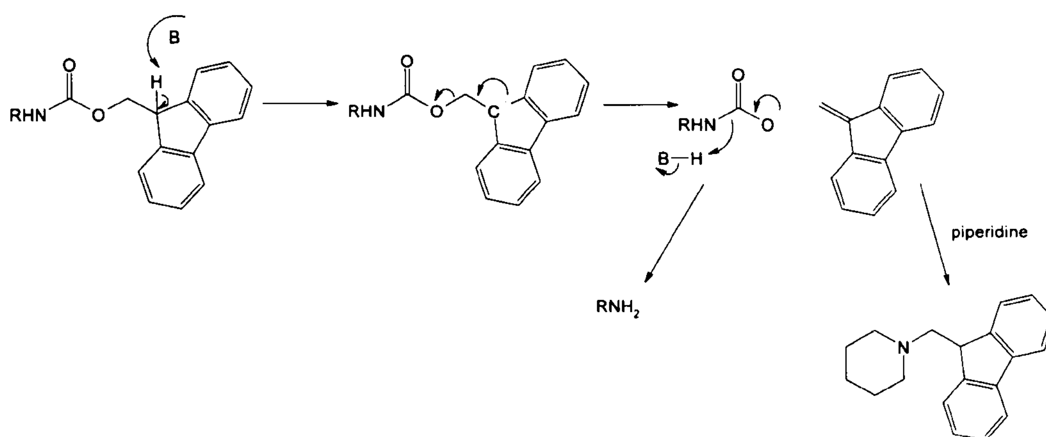


Figure 5-22 Removal of Fmoc protecting groups with piperidine.

Standard procedures for effecting the chain elongation of the peptide were successful with a PyBOP/HOBt system[267,272,*] being utilised for protected peptides and a HBTU/HOBt system[†266] for non-protected peptides. Standard methods of peptide cleavage from the resin were successful with 1% TFA in DCM being used for the protected peptide and 95% TFA with 2.5% TFA and 2.5% water being used for the peptide synthesised on a wang resin. The cleavage cocktail successfully removed the side chains of peptides and left the 4-Bbs protecting group on arginine intact.

All target peptides were successfully synthesised and utilised to synthesise a polymer library.

---

[*] 5 eq. of the amino acid, 4.9 eq. PyBOP, 5 eq. HOBt, 10 eq. DIPEA and 10ml DMF per gram of resin added to resin pre-swollen with DMF and purged with nitrogen for 24 hours, allowed to react for 1 hour and then washed with 3 aliquots of DMF.

[†] 5 eq. of the amino acid, 5 eq. HBTU, 5 eq. HOBt, 10 eq. DIPEA and 10ml DMF per gram of resin added to resin pre-swollen with DMF and purged with nitrogen for 24 hours, allowed to react for 1 hour and then washed with 3 aliquots of DMF. The mechanism of HBTU/HOBt mediated coupling is shown in Figure 5-23 below.

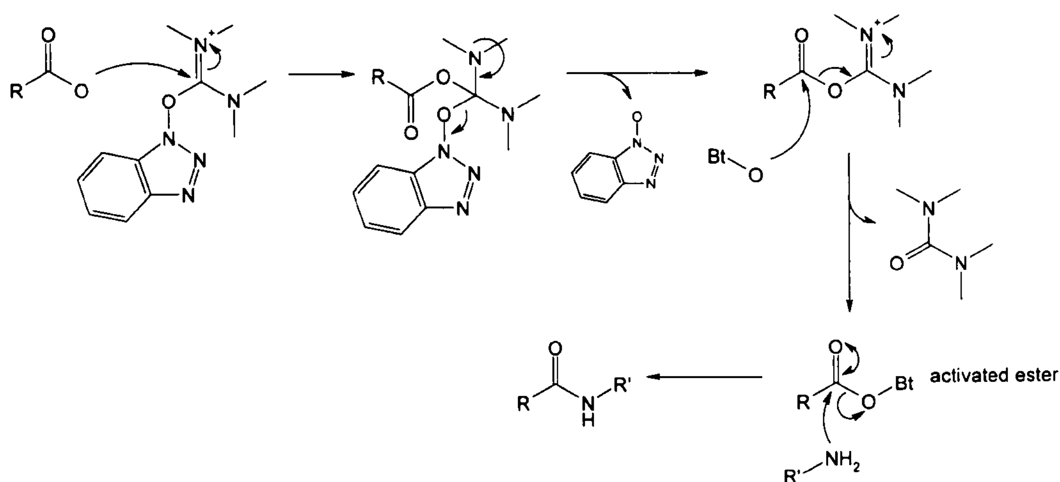


Figure 5-23 Reaction Scheme for HBTU mediated coupling of amino acids

5.5 Polymer Library Synthesis and Deprotection

5.5.1 Strategy 1: TFA Deprotection of Peptides

The UV initiation method of creating thin films could not be utilised with the TFA deprotection strategy as a suitable material on which to create the thin films could not be found. PET and other polymer sheets were not stable to TFA reaction and hence could not be used. Glass sheets could be used to make the polymers on but could not be cut into suitable shapes for cell culture. Glass microscope slides and coverslips of suitable shapes and sizes for placing in culture wells were investigated however the thin film could not be properly drawn over the glass due to the small size of the glass shapes. Consequently polymers containing GMAc and 2.5 wt % DVB were synthesised by the thermal polymerisation method described in section 7.2.3.

Disks were cut out from the sheet polymer and were washed three times with ethanol before being placed in 100% TFA for 4 hours. Upon removal from the TFA the disks were washed a further three times with ethanol to remove all traces of the acid. The transferral of the disks from ethanol to TFA resulted in a rapid size change in the polymers and the disks formed multiple hairline cracks or broke up entirely. The multiple hairline cracks resulted in a loss of transparency making the polymers unsuitable for cell culture as it would not be possible to visualise the morphology of the cells. The polymer disks were dried in a vacuum dessicator overnight and the experiment was repeated however the drying process also caused the polymer disks to break up. As a result of the fragmentation of the polymers this strategy of producing polymers incorporating

RGD was abandoned and efforts were concentrated on the second strategy of producing the peptide with enzymatically cleavable protecting groups.

5.5.2 Strategy 2: Enzyme Deprotection

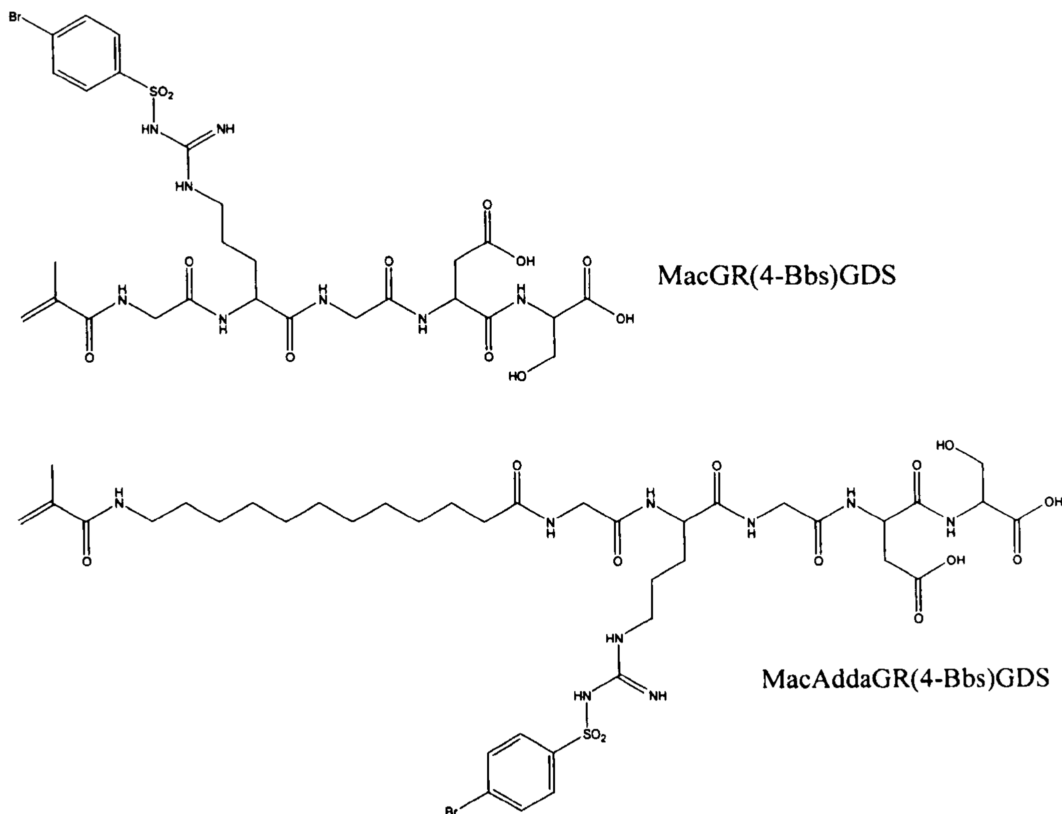


Figure 5-24 Target peptides for use with strategy 2: Enzyme deprotection

The target peptides (shown above in Figure 5-24) were synthesised as described in section 7.5 and stock solutions containing 0.01, 0.1, 1 and 10 μmol of peptide per millilitre of methanol were prepared. Stock solutions containing monomer, 2.5 wt % of EGDMA and 1 wt % of HMPP were also prepared. Polymerisation solutions were made by combining a known volume of monomer stock solution with known volume of peptide stock solution. The volumes were adjusted to allow for the different densities of the solutions such that the weight percentages remained constant. Thin films were then created by the techniques described in section 7.7.

Disks of an appropriate size were cut out from the thin films and placed inside culture wells in a sterile culture hood. The polymers were washed with ethanol three times to sterilize the polymers and to remove soluble impurities from the hydrogel such as unreacted initiator. All polymers were then washed three times with PBS to remove ethanol from the hydrogel. Those polymers that

required deprotection with the enzyme had 0.5ml of the GST incubation solution added to them and then they were stored for 24 hours in an incubator at 37°C with 5% CO_2. Polymers that were incubated for more than one day had the enzyme solution changed at 24 hour intervals.

Tables showing the polymers synthesised and the deprotection procedures are shown below. Each polymer was given a code that referred to its composition and treatment with four parts indicating the value of each variable such as G-S-10-1. The first letter refers to the monomer type (GMMA = G, BMA = B), the second letter refers to the peptide sequence (S = short, without spacer arm, L = long, with spacer arm), the first number refers to the nominal concentration of peptide in μmol/g and the last number refers to the number of days pre-treatment with GST.

Polymers synthesised with the peptide sequence MacGR(4-Bbs)GDS

Polymer Code	Composition wt % GMMA: BMA: EGDMA	Nominal Peptide Concentration / µmol g^{-1}	Enzyme Treatment
GE-S-10-0	100: 0: 2.5	10	No treatment
GE-S-10-1	100: 0: 2.5	10	1 day GST/GSH
GE-S-10-3	100: 0: 2.5	10	3 days GST/GSH
GE-S-10-5	100: 0: 2.5	10	5 days GST/GSH
GE-S-1-0	100: 0: 2.5	1	No treatment
GE-S-1-1	100: 0: 2.5	1	1 day GST/GSH
GE-S-1-3	100: 0: 2.5	1	3 days GST/GSH
GE-S-1-5	100: 0: 2.5	1	5 days GST/GSH
GE-S-0.1-0	100: 0: 2.5	0.1	No treatment
GE-S-0.1-1	100: 0: 2.5	0.1	1 day GST/GSH
GE-S-0.1-3	100: 0: 2.5	0.1	3 days GST/GSH
GE-S-0.1-5	100: 0: 2.5	0.1	5 days GST/GSH
GE-S-0.01-0	100: 0: 2.5	0.01	No treatment
GE-S-0.01-1	100: 0: 2.5	0.01	1 day GST/GSH
GE-S-0.01-3	100: 0: 2.5	0.01	3 days GST/GSH
GE-S-0.01-5	100: 0: 2.5	0.01	5 days GST/GSH
BE-S-10-0	0: 100: 2.5	10	No treatment
BE-S-10-1	0: 100: 2.5	10	1 day GST/GSH
BE-S-10-3	0: 100: 2.5	10	3 days GST/GSH
BE-S-10-5	0: 100: 2.5	10	5 days GST/GSH
BE-S-1-0	0: 100: 2.5	1	No treatment
BE-S-1-1	0: 100: 2.5	1	1 day GST/GSH
BE-S-1-3	0: 100: 2.5	1	3 days GST/GSH
BE-S-1-5	0: 100: 2.5	1	5 days GST/GSH
BE-S-0.1-0	0: 100: 2.5	0.1	No treatment
BE-S-0.1-1	0: 100: 2.5	0.1	1 day GST/GSH
BE-S-0.1-3	0: 100: 2.5	0.1	3 days GST/GSH
BE-S-0.1-5	0: 100: 2.5	0.1	5 days GST/GSH
BE-S-0.01-0	0: 100: 2.5	0.01	No treatment
BE-S-0.01-1	0: 100: 2.5	0.01	1 day GST/GSH
BE-S-0.01-3	0: 100: 2.5	0.01	3 days GST/GSH
BE-S-0.01-5	0: 100: 2.5	0.01	5 days GST/GSH

Table 5-6 Polymers synthesised with the short peptide sequence Mac-GR(4-Bbs)GDS and treated with the enzyme GST to remove protecting group on arginine. Treatment with GST/GSH comprised of incubating polymer disks in individual wells of a tissue culture plate with 250µl of a solution of 0.05mg/ml GST and 500µM GSH in PBS (pH 7.4) at 37°C. Where the treatment was conducted for more than 1 day the solution was changed on a daily basis.

Polymers synthesised with the peptide sequence MacAddaGR(4-Bbs)GDS

Polymer Code	Composition wt % GMMA: BMA: EGDMA	Nominal Peptide Concentration / μmol g⁻	Enzyme Treatment
GE-L-10-0	100: 0: 2.5	10	No treatment
GE-L-10-1	100: 0: 2.5	10	1 day GST/GSH
GE-L-10-3	100: 0: 2.5	10	3 days GST/GSH
GE-L-10-5	100: 0: 2.5	10	5 days GST/GSH
GE-L-1-0	100: 0: 2.5	1	No treatment
GE-L-1-1	100: 0: 2.5	1	1 day GST/GSH
GE-L-1-3	100: 0: 2.5	1	3 days GST/GSH
GE-L-1-5	100: 0: 2.5	1	5 days GST/GSH
GE-L-0.1-0	100: 0: 2.5	0.1	No treatment
GE-L-0.1-1	100: 0: 2.5	0.1	1 day GST/GSH
GE-L-0.1-3	100: 0: 2.5	0.1	3 days GST/GSH
GE-L-0.1-5	100: 0: 2.5	0.1	5 days GST/GSH
GE-L-0.01-0	100: 0: 2.5	0.01	No treatment
GE-L-0.01-1	100: 0: 2.5	0.01	1 day GST/GSH
GE-L-0.01-3	100: 0: 2.5	0.01	3 days GST/GSH
GE-L-0.01-5	100: 0: 2.5	0.01	5 days GST/GSH
BE-L-10-0	0: 100: 2.5	10	No treatment
BE-L-10-1	0: 100: 2.5	10	1 day GST/GSH
BE-L-10-3	0: 100: 2.5	10	3 days GST/GSH
BE-L-10-5	0: 100: 2.5	10	5 days GST/GSH
BE-L-1-0	0: 100: 2.5	1	No treatment
BE-L-1-1	0: 100: 2.5	1	1 day GST/GSH
BE-L-1-3	0: 100: 2.5	1	3 days GST/GSH
BE-L-1-5	0: 100: 2.5	1	5 days GST/GSH
BE-L-0.1-0	0: 100: 2.5	0.1	No treatment
BE-L-0.1-1	0: 100: 2.5	0.1	1 day GST/GSH
BE-L-0.1-3	0: 100: 2.5	0.1	3 days GST/GSH
BE-L-0.1-5	0: 100: 2.5	0.1	5 days GST/GSH
BE-L-0.01-0	0: 100: 2.5	0.01	No treatment
BE-L-0.01-1	0: 100: 2.5	0.01	1 day GST/GSH
BE-L-0.01-3	0: 100: 2.5	0.01	3 days GST/GSH
BE-L-0.01-5	0: 100: 2.5	0.01	5 days GST/GSH

Table 5-7 Polymers synthesised with the long peptide sequence Mac-Adda-GR(4-Bbs)GDS and treated with the enzyme GST to remove protecting group on arginine. Treatment with GST/GSH comprised of incubating polymer disks in individual wells of a tissue culture plate with 250μl of a solution of 0.05mg/ml GST and 500μM GSH in PBS (pH 7.4) at 37°C. Where the treatment was conducted for more than 1 day the solution was changed on a daily basis.

5.6 Analysis of Cell Behaviour on Polymers

The aims of the cell culture on the polymer are to both qualitatively and quantitatively determine the ability of each polymer to promote cell adhesion and function. This shall be done in two main ways, the use of the MTT assay to quantitatively determine cell activity levels and visual methods to determine the morphology of the cells. Cellular adhesion occurs primarily in the first 10 hours after the cells are plated on a material however the purpose of this study is to analyse both the adhesion and the normal function of the cells so it was decided to use a cell culture time of 24 hours prior to analysis of the cells.

5.6.1 General Information

The library contained 4 different variables; peptide concentration, peptide type, monomer type and number of days deprotection. The peptide concentration variable investigated a total of 5 different concentrations 10mmol, 1mmol, 0.1mmol, 0.01μmol/g and no peptide. The two different peptide types were target peptide S (short - without a spacer arm) and target peptide L (long - with a spacer arm). The monomer types were butyl methacrylate and glycerol methacrylate. The cross-linker concentration was kept constant at 2.5 wt %. As it is unknown whether the sulphonamide side chain had been deprotected by the enzyme solution it was decided initially to examine the effect of 0, 1 and 3 days deprotection on the library of hydrogels. In total the library consisted of 60 different polymers. Students non-paired T-test assuming non-equal variances with a one-tailed distribution was utilised to calculate significances.

5.6.2 Serum-Containing Media

The photographs below show the morphology of the cells on the control substrates that do not contain RGD; poly(GMMA-co-EGDMA) (abbreviated to GE), poly(BMA-co-EGDMA) (abbreviated to BE) and TCP after 4 and 24 hours.

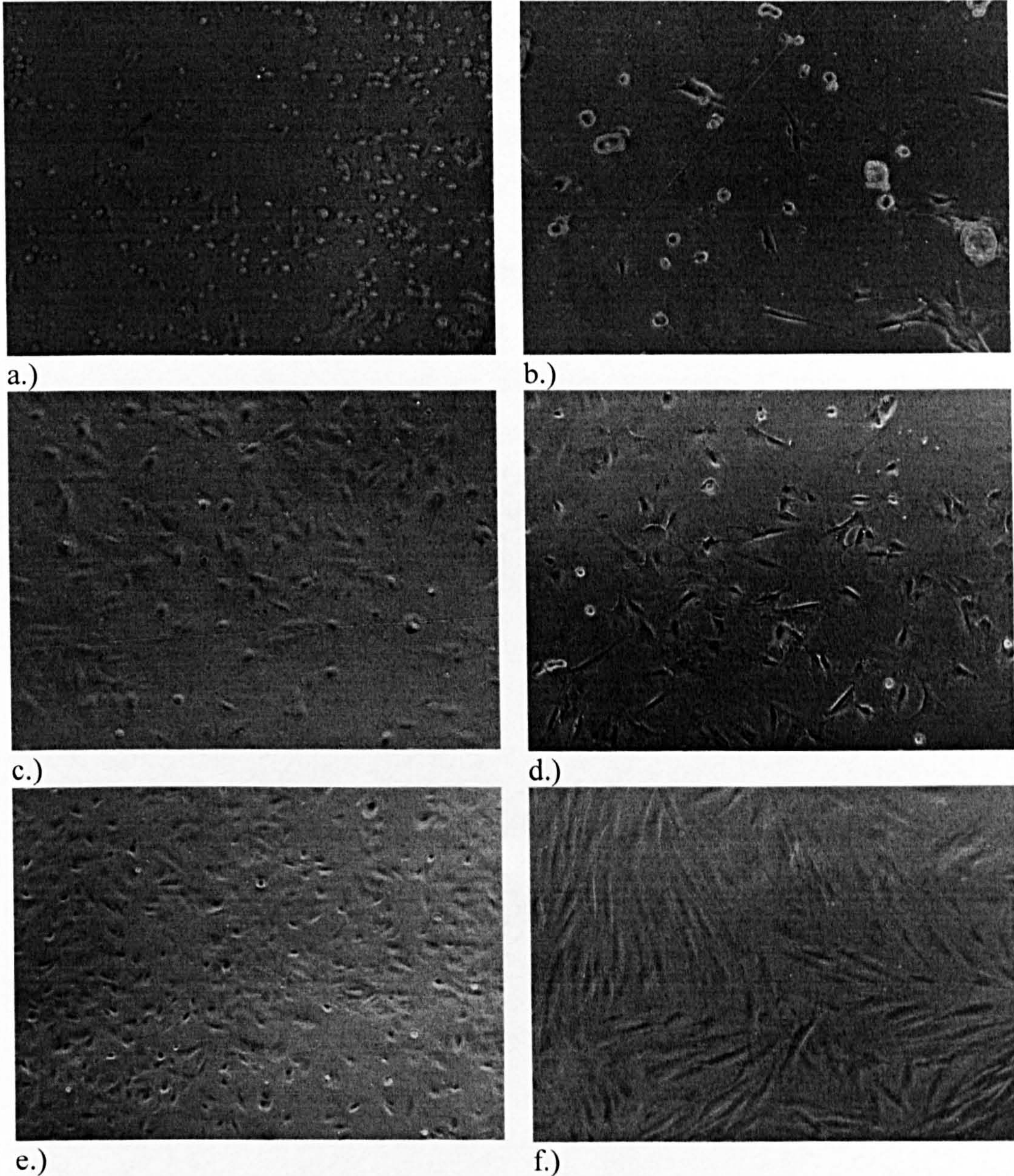


Figure 5-25 Primary human dermal fibroblasts (F1833, passage 10) cultured in media containing 10% FCS on a.) GE 4hrs, b.) GE 24 hours c.) BE 4 hrs d.) BE 24hrs, e.) TCP 4 hrs and f.) TCP 24hrs

After 4 hours the cells have started to attach to the surfaces but have not yet fully spread and obtained their final morphologies. On glycerol methacrylate containing surfaces the cells adhere poorly and after 4 hours, still retain the unnatural spherical morphology created by the harvesting of the cells from the surface on which they were amplified. After 24 hours some of the cells have

managed to spread on the glycerol methacrylate surfaces however the majority remain rounded and some have aggregated into large cellular bundles. Cells on the butyl methacrylate surfaces appear to adhere well after 4 hours but the cell spreading discontinues and the cells are not much better spread after 24 hours. In contrast to this on tissue culture plastic the cells adhere and spread as well to begin with and the spreading continues over the 24 hour time period such that, with this culture area and cell seeding density, the cells have formed a confluent monolayer of elongated cells. The polymers without RGD do exhibit a range of cellular behaviours according to whether the substrate can adsorb proteins.

5.6.2.1 GMMA Polymers containing Long Peptide Sequence

The effect of the incorporation of RGD should improve the glycerol methacrylate polymers as there are no competing processes of protein adsorption and there is a large area for improvement in the cell morphology.

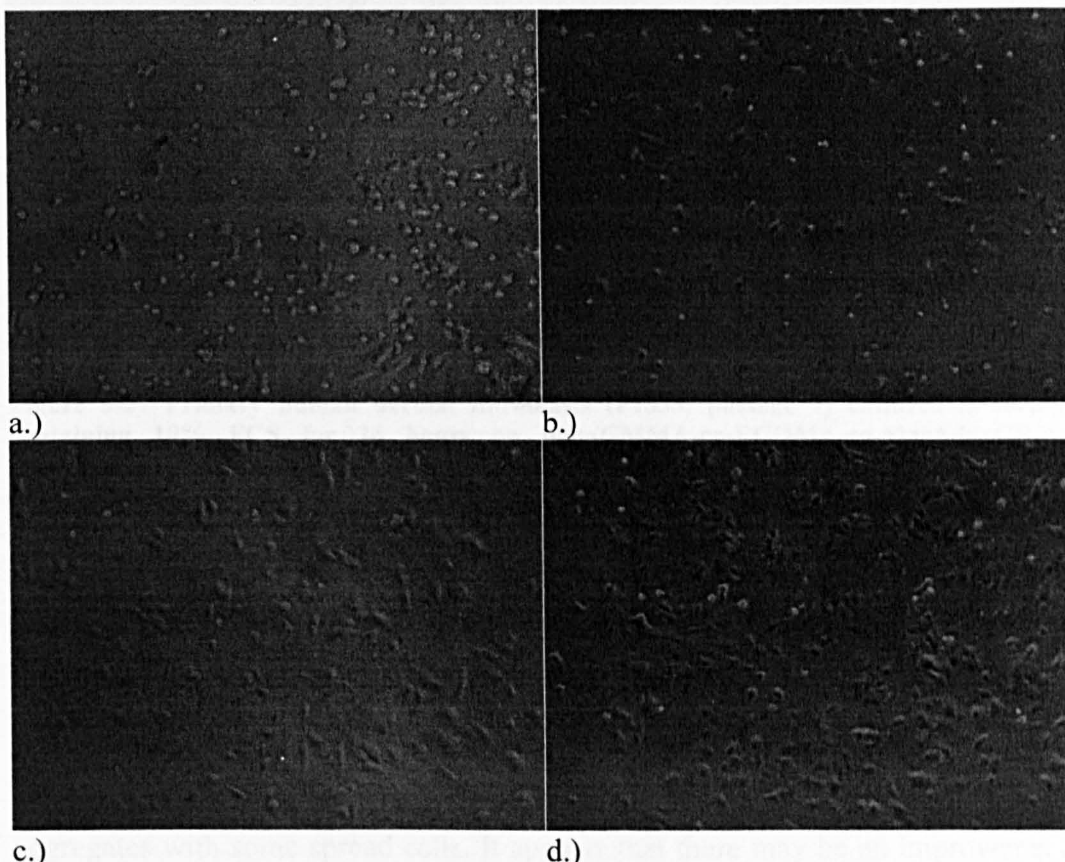


Figure 5-26 Primary human dermal fibroblasts (F1833, passage 7) cultured in media containing 10% FCS for 4 hours on poly(GMMA-co-EGDMA-co-MacAddaGR(4-Bbs)GDS) pre-incubated with 0.05 mg/,ml GST and 500 μmol dm^{-3} GSH for 1 day a.) 0.01 μmol/g peptide, b.) 0.1 μmol/g peptide, c.) 1 μmol/g peptide and d.) 10 μmol/g peptide

After 4 hours there are already large differences between the way cells behave on polymers containing different concentrations of RGD as shown in Figure 5-26 (p103). At the lowest RGD concentration cells appear very similar to those on the blank hydrogel whereas at the higher concentrations the cells appear more like those on tissue culture plastic at the same time.

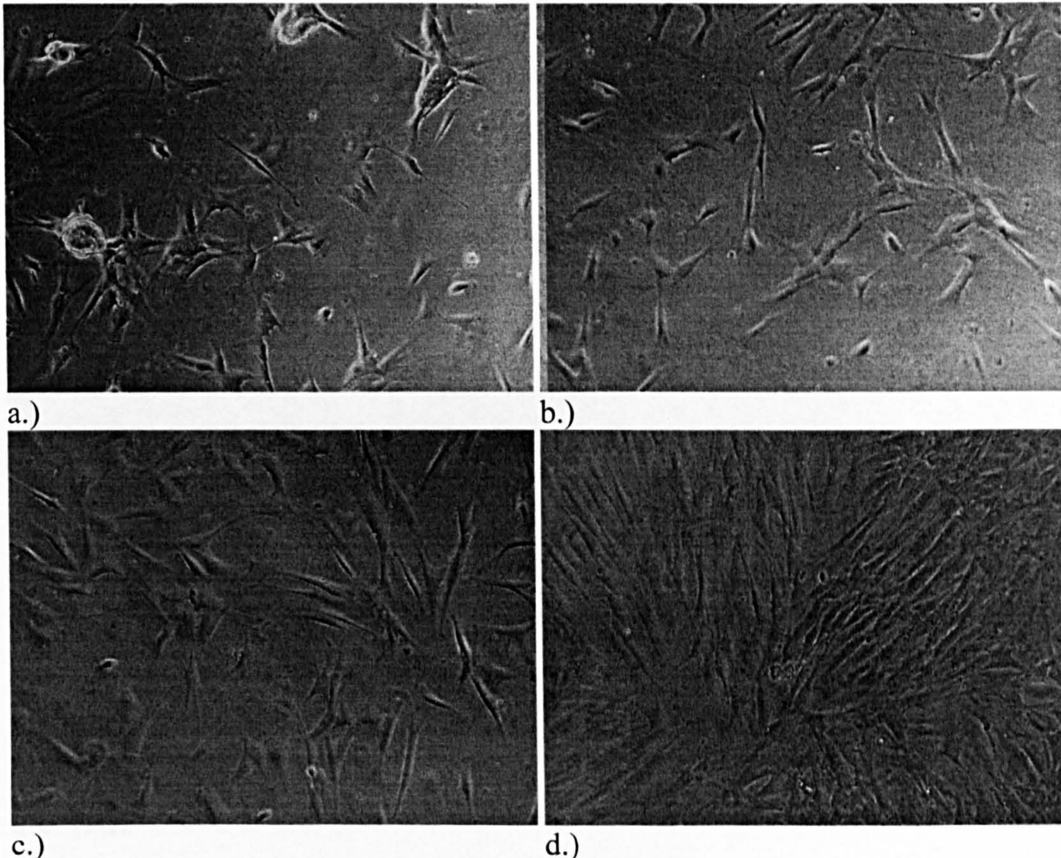


Figure 5-27 Primary human dermal fibroblasts (F1833, passage 7) cultured in media containing 10% FCS for 24 hours on poly(GMMA-co-EGDMA-co-MacAddaGR(4-Bbs)GDS) pre-incubated with 0.05 mg/,ml GST and 500 μmol dm^{-3} GSH for 1 day a.) 0.01 μmol/g peptide, b.) 0.1 μmol/g peptide, c.) 1 μmol/g peptide and d.) 10 μmol/g peptide

After 24 hours the cells show the same trends in improving cell morphologies as the peptide concentration increases. Over the range of 0.01 to 10 μmol / g there is a continual improvement in cell morphology and cell density. The cells cultured on the 0.01 mmol /g polymer (a. in Figure 5-27 above) show similar features to those on the blank polymer (d. in Figure 5-26) of cellular aggregates with some spread cells. It appears that there may be an improvement in cell number per field of view however this is difficult to determine. Both polymers with intermediate concentrations of RGD show definite improvements in cell morphology and the polymer with the highest concentration appeared indistinguishable from the positive control, TCP. These photographs clearly

show that there is a definite improvement in the abilities of polymers to adhere cells as the concentration of the peptide increases.

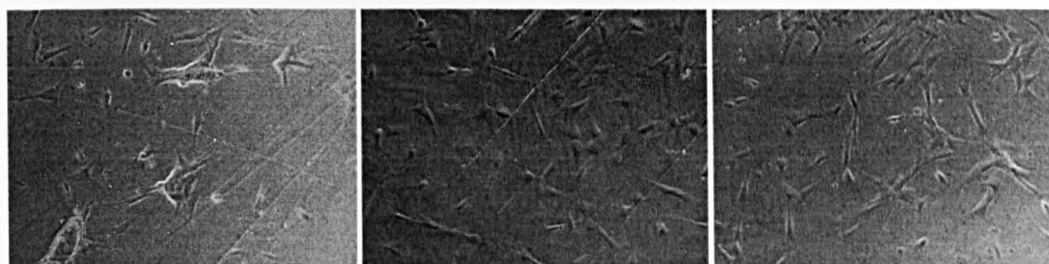
The synthesis route to the incorporated peptide required the use of the enzyme Glutathione-S-Transferase to deprotect the sulphonyl group on the arginine. The protected peptide will not be able to bind to integrins as the size and electrostatic properties of the sequence will have been masked by the protecting group. Consequently polymers containing peptide that have not been deprotected ought to exhibit cell morphologies similar to that of the background substrate glycerol methacrylate.

The morphologies of cells on polymers containing different levels of RGD and different number of days deprotection with the enzyme GST was investigated.

a.) b.) c.)

Figure 5-28 Primary human dermal fibroblasts (F1833, passage 7) cultured in media containing 10% FCS for 24 hours on poly(GMMA-co-EGDMA) containing 0.01 μmol/g MacAddaGR(4-Bbs)GDS a.) no GST deprotection, b.) 1 days incubation with 0.05 mg/,ml GST and 500 μmol dm^{-3} GSH and c.) 3 days incubation with 0.05 mg/,ml GST and 500 μmol dm^{-3} GSH

After 24 hours the morphologies of all cells on polymers containing the minimal concentration of RGD of 0.01μmol/g were similar both to each other and to the background substrate.



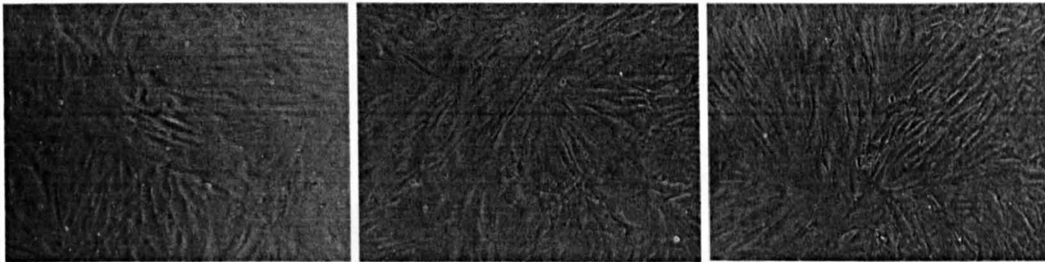
a.) b.) c.)

Figure 5-29 Primary human dermal fibroblasts (F1833, passage 7) cultured in media containing 10% FCS for 24 hours on poly(GMMA-co-EGDMA) containing 0.1 μmol/g MacAddaGR(4-Bbs)GDS a.) no GST deprotection, b.) 1 days incubation with 0.05 mg/,ml GST and 500 μmol dm^{-3} GSH and c.) 3 days incubation with 0.05 mg/,ml GST and 500 μmol dm^{-3} GSH

The non-deprotected peptide at a concentration of 0.1 µmol / g appeared to be ineffective at binding cells and the morphologies looked similar to those of the background substrate with some large cellular aggregates and some spread cells. In contrast those polymers in which the peptide had been deprotected by GST for either one or three days showed better cell spreading than in that of the background substrate.

a.) b.) c.)

Figure 5-30 Primary human dermal fibroblasts (F1833, passage 7) cultured in media containing 10% FCS for 24 hours on poly(GMMA-co-EGDMA) containing 1 µmol/g MacAddaGR(4-Bbs)GDS a.) no GST deprotection, b.) 1 days incubation with 0.05 mg/,ml GST and 500 µmol dm^{-3} GSH and c.) 3 days incubation with 0.05 mg/,ml GST and 500 µmol dm^{-3} GSH



a.) b.) c.)

Figure 5-31 Primary human dermal fibroblasts (F1833, passage 7) cultured in media containing 10% FCS for 24 hours on poly(GMMA-co-EGDMA) containing 10 µmol/g MacAddaGR(4-Bbs)GDS a.) no GST deprotection, b.) 1 days incubation with 0.05 mg/,ml GST and 500 µmol dm^{-3} GSH and c.) 3 days incubation with 0.05 mg/,ml GST and 500 µmol dm^{-3} GSH

With a concentration of 1 or 10 µmol/g of peptide incorporated into the glycerol methacrylate polymer the cells appear similar on all polymers regardless of whether the deprotection has been performed on the substrate prior to cell culture.

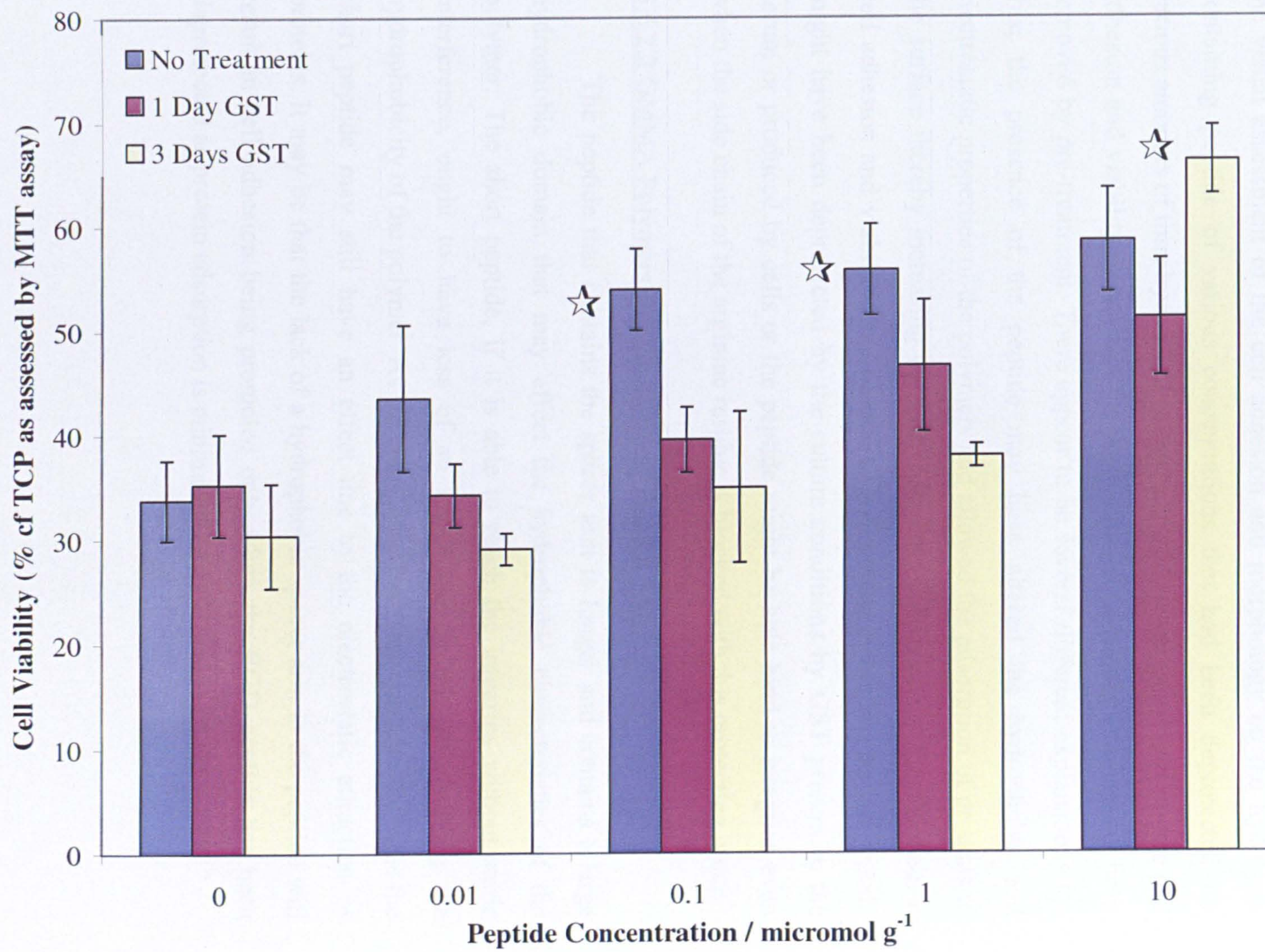


Figure 5-32 Viability of human dermal fibroblasts cultured for 24 hours in media containing 10% FCS on glycerol methacrylate polymers containing varying concentrations of MacAddaGR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates errors shown are the standard deviation divided by the number of replicates. Polymers were deprotected with 0.05mg/ml GST and 500 µmol dm^{-3} GSH in PBS and 0.5ml per sample for 0, 1 or 3 days (deprotection solution was replaced daily). Viability of cells (F1833, passage 7) was assessed via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the non-peptide containing control that has been treated with the same amount of GST (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

The MTT assay results shown in Figure 5-32 support the trends inferred by the photographic evidence that the cell culturing ability of the polymers increases as the peptide concentration increases. They also show that the pre-treatment of the peptide with GST does not in fact increase the cell viability as, in many cases, the non-treated peptide containing polymers give a higher MTT value and a more significant improvement in cell adhesion and viability compared to the non-peptide containing control. The MTT assay results confirm the visual assessment of the cell adhesion and morphology on the hydrogels containing peptide of various concentrations that had been deprotected for various amounts of time by GST. It is apparent that the peptide does promote cell adhesion and viability even when the protecting group on arginine has not been removed by pre-treatment. There appear to be several different explanations for this; the presence of the peptide may have altered the hydrophobic and electrostatic properties of the polymers and allowed the adsorption of proteins to the surface thereby increasing the cell spreading. Alternatively the increase in cell adhesion and viability may be due to RGD-integrin binding as the peptide might have been deprotected by the culture conditions by GST present in the serum or produced by cells or the peptide might be able bind to integrins even when the side chain of the arginine residue is blocked with this protecting group.

5.6.2.2 GMMA Polymers containing Short Peptide Sequence

The peptide that contains the spacer arm is longer and contains a large hydrophobic domain that may affect the hydrophobic characteristics of the polymer. The short peptide, if it is able to reach the integrins without steric interference, ought to have less of an effect that is due to changing the hydrophobicity of the polymer. As the charges on both polymers are the same the short peptide may still have an effect due to the electrostatic attraction of proteins. It may be that the lack of a hydrophobic spacer arm in the peptide will result in cell adhesion being promoted only when the RGD peptide has been deprotected as protein adsorption is minimised.

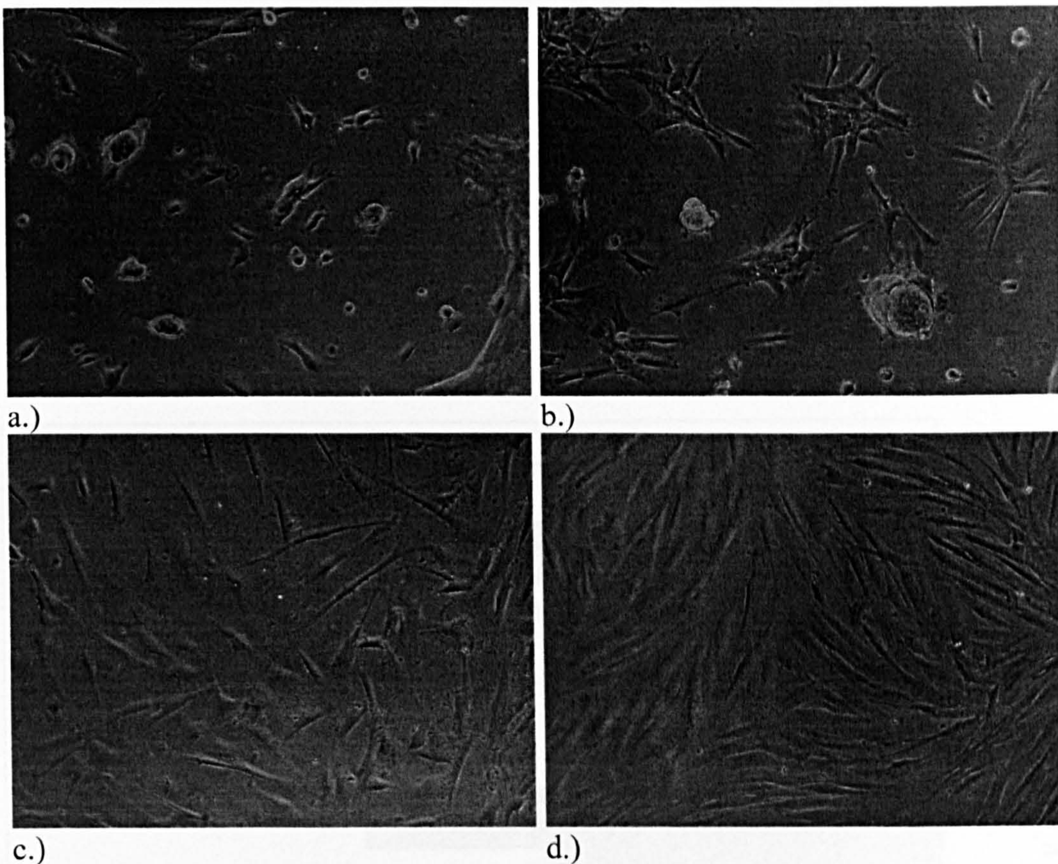


Figure 5-33 Primary human dermal fibroblasts (F1833, passage 7) cultured in media containing 10% FCS for 24 hours on poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) pre-incubated with 0.05 mg/,ml GST and 500 μmol dm^{-3} GSH for 1 day a.) 0.01 μmol/g peptide, b.) 0.1 μmol/g peptide, c.) 1 μmol/g peptide and d.) 10 μmol/g peptide

After 24 hours culture of cells on polymer containing the short peptide sequence the cells appeared to be well spread on those polymers containing high concentrations of peptide and less well spread as the concentration decreased. This follows the same trends as observed with the long peptide sequence. AS in the previous set of results there were no significant differences observed between deprotected and non-treated materials.

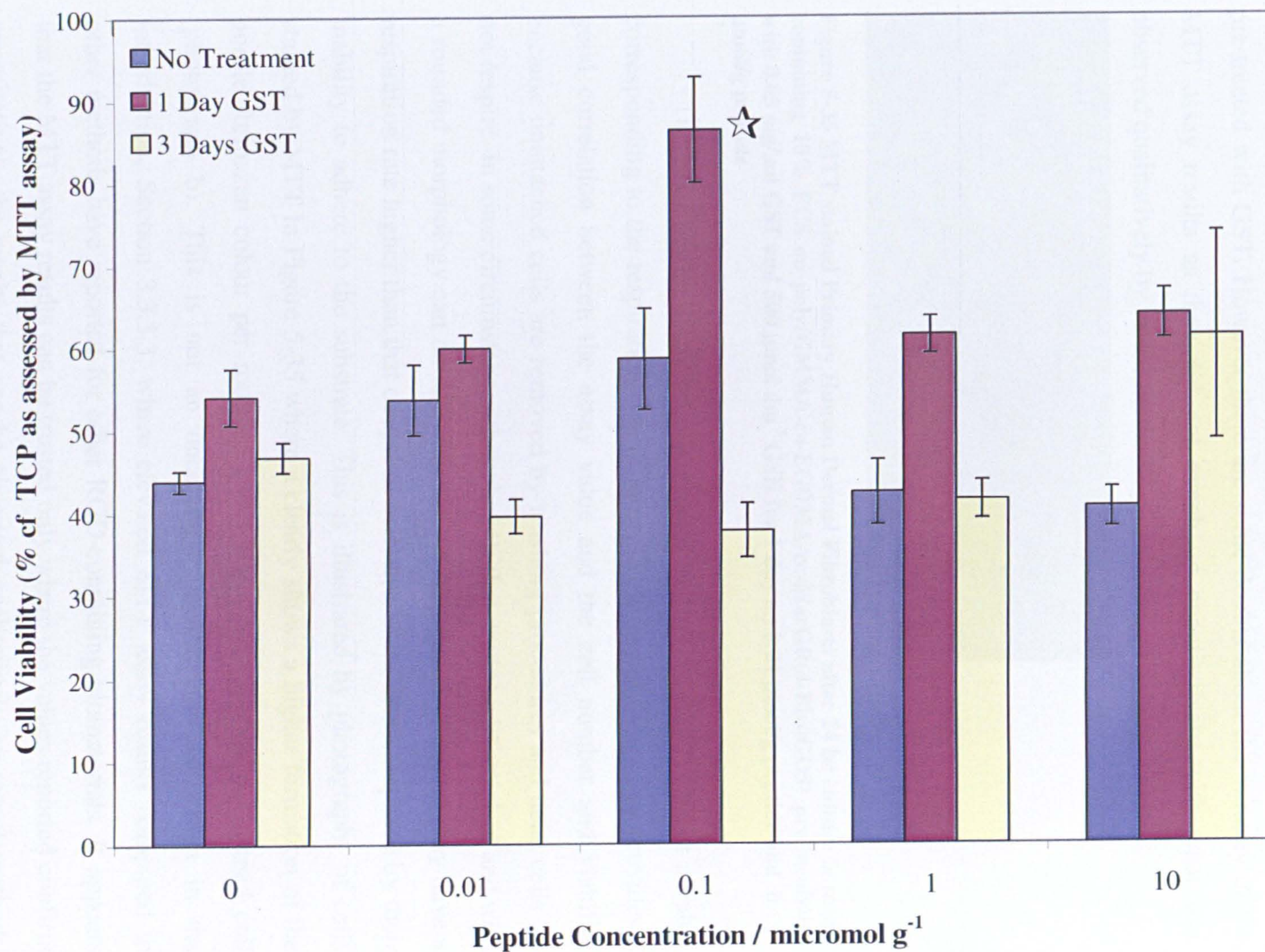


Figure 5-34 Viability of human dermal fibroblasts cultured for 24 hours in media containing 10% FCS on glycerol methacrylate polymers containing varying concentrations of MacGR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates and errors shown are the standard deviation divided by the number of replicates. Polymers were deprotected with 0.05mg/ml GST and 500µmol dm^{-3} GSH in PBS and 0.5ml per sample for 0, 1 or 3 days (deprotection solution was replaced daily). Viability of cells (F1833. passage 7) was assessed via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the non-peptide containing control that has been treated with the same amount of GST (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

The MTT assay results for polymers containing the short polymer sequence are less clear cut that those for the long polymer sequence. Certainly the incorporation of 0.1μmol/g MacGR(4-Bbs)GDS followed by 1 days incubation with GST resulted in a statistically significant increase in cell viability as assessed by the MTT assay. There may be a trend towards increasing assay value as the concentration increases however this is certainly less pronounced than previously and is not statistically significant. These results do show that there is no improvement in viability gained when the protected peptide has been pre-treated with GST. However there are some doubts about the veracity of the MTT assay results as they did not match up quantitatively with what was observed qualitatively by visual methods.

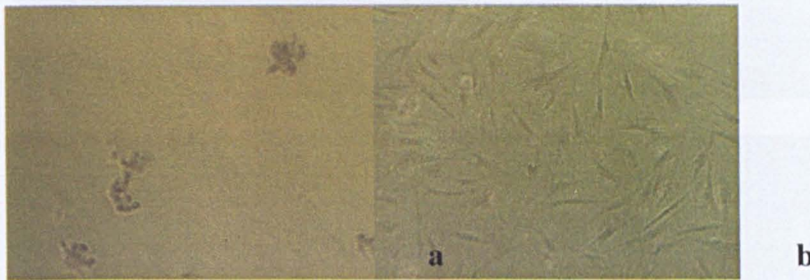


Figure 5-35 MTT stained Primary Human Dermal Fibroblasts after 24 hr culture in media containing 10% FCS on poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) pre-incubated with 0.05 mg/,ml GST and 500 μmol dm^{-3} GSH for 1 day a.) 0.01 μmol/g peptide and b.) 10 μmol/g peptide

The MTT results must be treated with caution as they represent a value corresponding to the respiration rate of the cells. In general the assay provides a good correlation between the assay value and the cell number and viability because unattached cells are removed by washing procedures and dead cells do not respire. In some circumstances though cells that are loosely attached and with a rounded morphology can remain after the washing procedures and may have a respiration rate higher than that of spread cells due to the stress imposed by their inability to adhere to the substrate. This is illustrated by photographs of cells stained by MTT in Figure 5-35 where a clearly shows a higher formation of the purple formazan colour per rounded cell (photograph a) than per spread cell (photograph b). This is not an uncommon phenomenon as shown in the introduction, Section 3.3.3.3, where elevated MTT assay results compared to other methods have reported for other RGD-containing biomaterials. It appears that the MTT assay results can be trusted only where the values reported confirm quantitatively the results that can be observed qualitatively by visual methods.

5.6.2.3 BMA Polymers containing Short and Long Peptide Sequences

Butyl methacrylate polymers allow the adsorption of proteins to the surface and consequently the RGD peptide may be masked by the covering proteins and its effects invisible or less pronounced than in the glycerol methacrylate polymers.

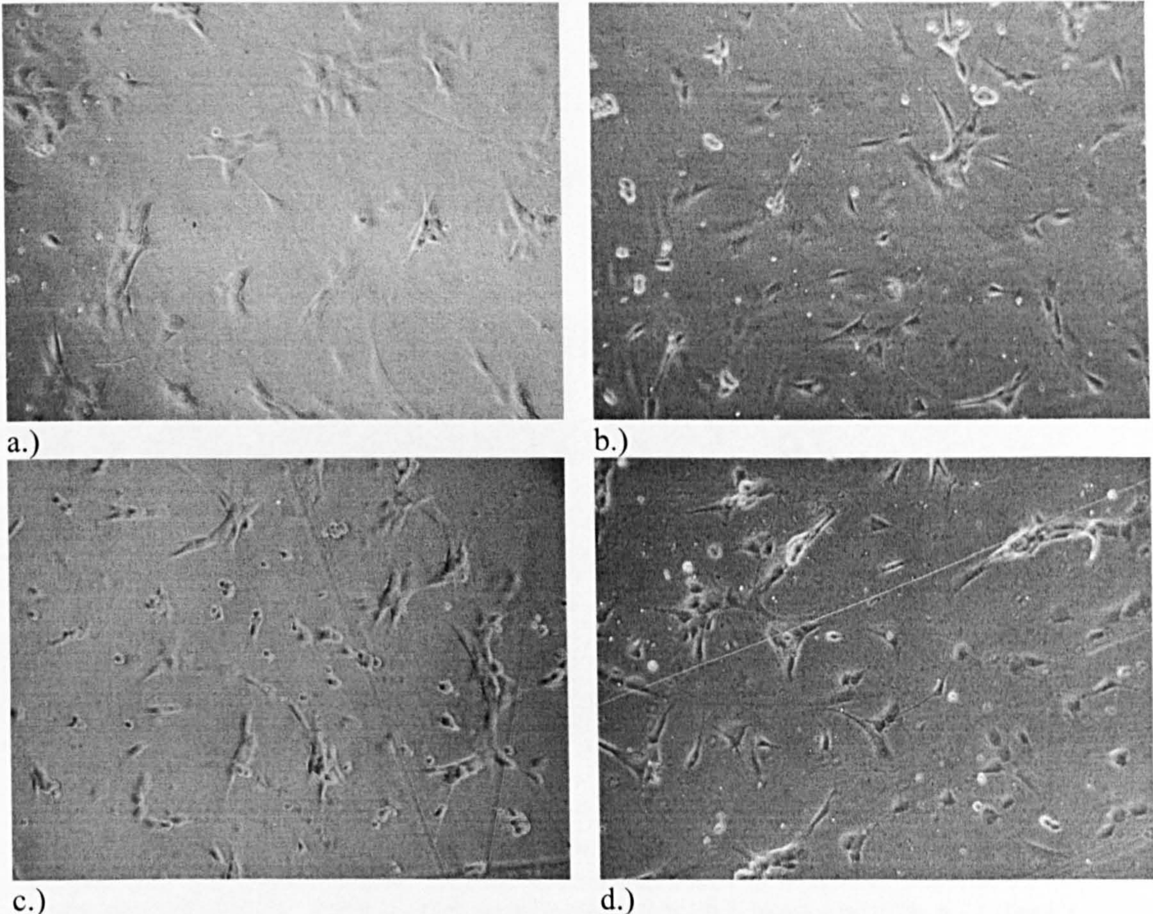
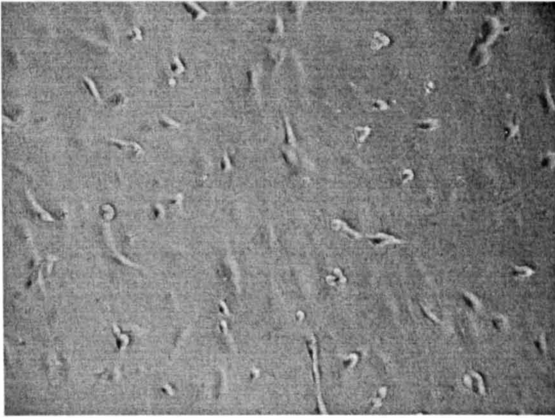
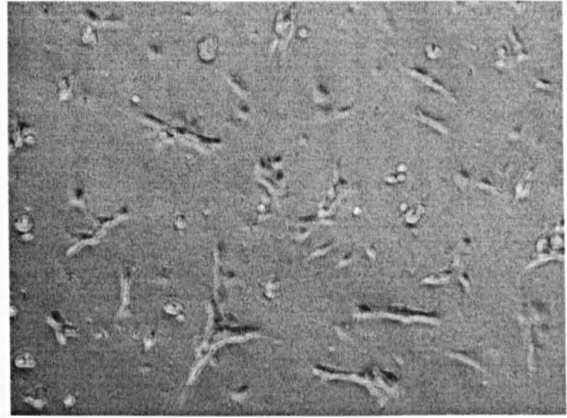


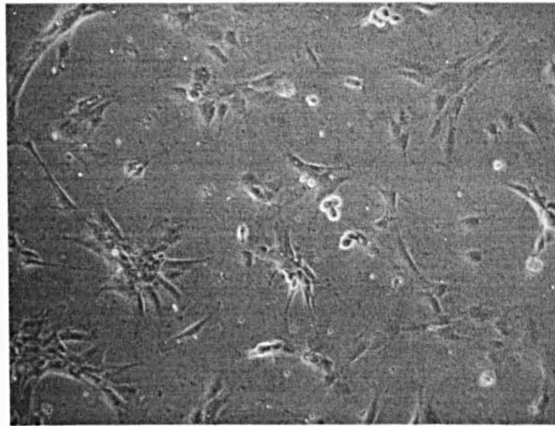
Figure 5-36 Primary human dermal fibroblasts (F1833, passage 10) cultured in media containing 10% FCS for 24 hours on poly(BMA-co-EGDMA-co-MacAddaGR(4-Bbs)GDS) pre-incubated with 0.05 mg/,ml GST and 500 μmol dm^{-3} GSH for 1 day a.) 0.01 μmol/g peptide, b.) 0.1 μmol/g peptide, c.) 1 μmol/g peptide and d.) 10 μmol/g peptide



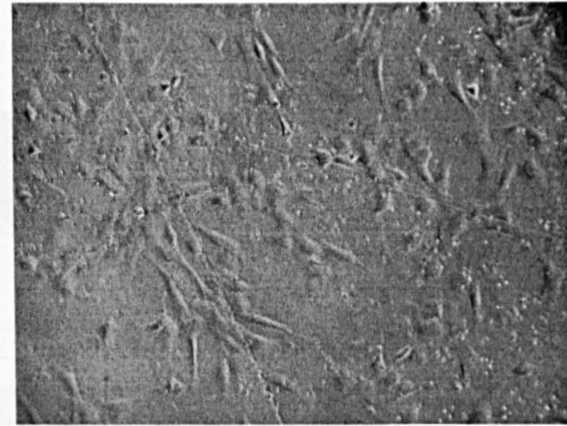
a.)



b.)



c.)



d.)

Figure 5-37 Primary human dermal fibroblasts (F1833, passage 10) cultured in media containing 10% FCS for 24 hours on poly(BMA-co-EGDMA-co-MacGR(4-Bbs)GDS) pre-incubated with GST for 1 day a.) 0.01 μmol/g peptide, b.) 0.1 μmol/g peptide, c.) 1 μmol/g peptide and d.) 10 μmol/g peptide

Figure 5-36 and Figure 5-37 above shows no differences in the cell morphologies after 24 hours between the polymers containing the long peptide sequence with the spacer arm and those with the peptide without the spacer arm at the same concentration. There is some evidence that cells are better spread on the polymers containing larger amounts of peptides but this is not drastic enough to be conclusive.

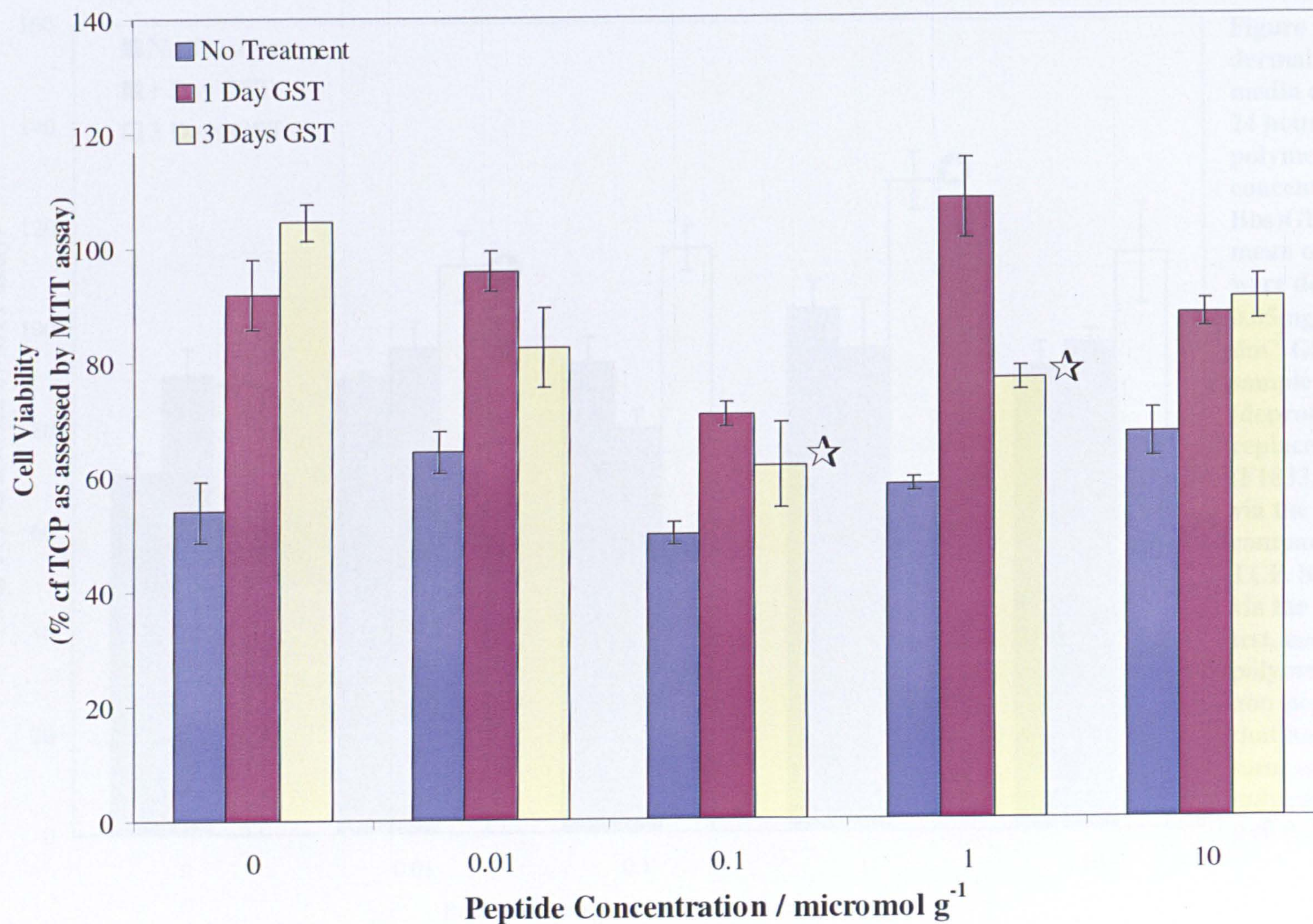


Figure 5-38 Viability of human dermal fibroblasts cultured for 24 hours in media containing 10% FCS on butyl methacrylate polymers containing varying concentrations of Mac-Adda-GR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates. Polymers were deprotected with 0.05mg/ml GST and 500µmol dm^{-3} GSH in PBS and 0.5ml per sample for 0, 1 or 3 days (deprotection solution was replaced daily). Viability of cells (F1833. passage 10) was assessed via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the non-peptide containing control that has been treated with the same amount of GST (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

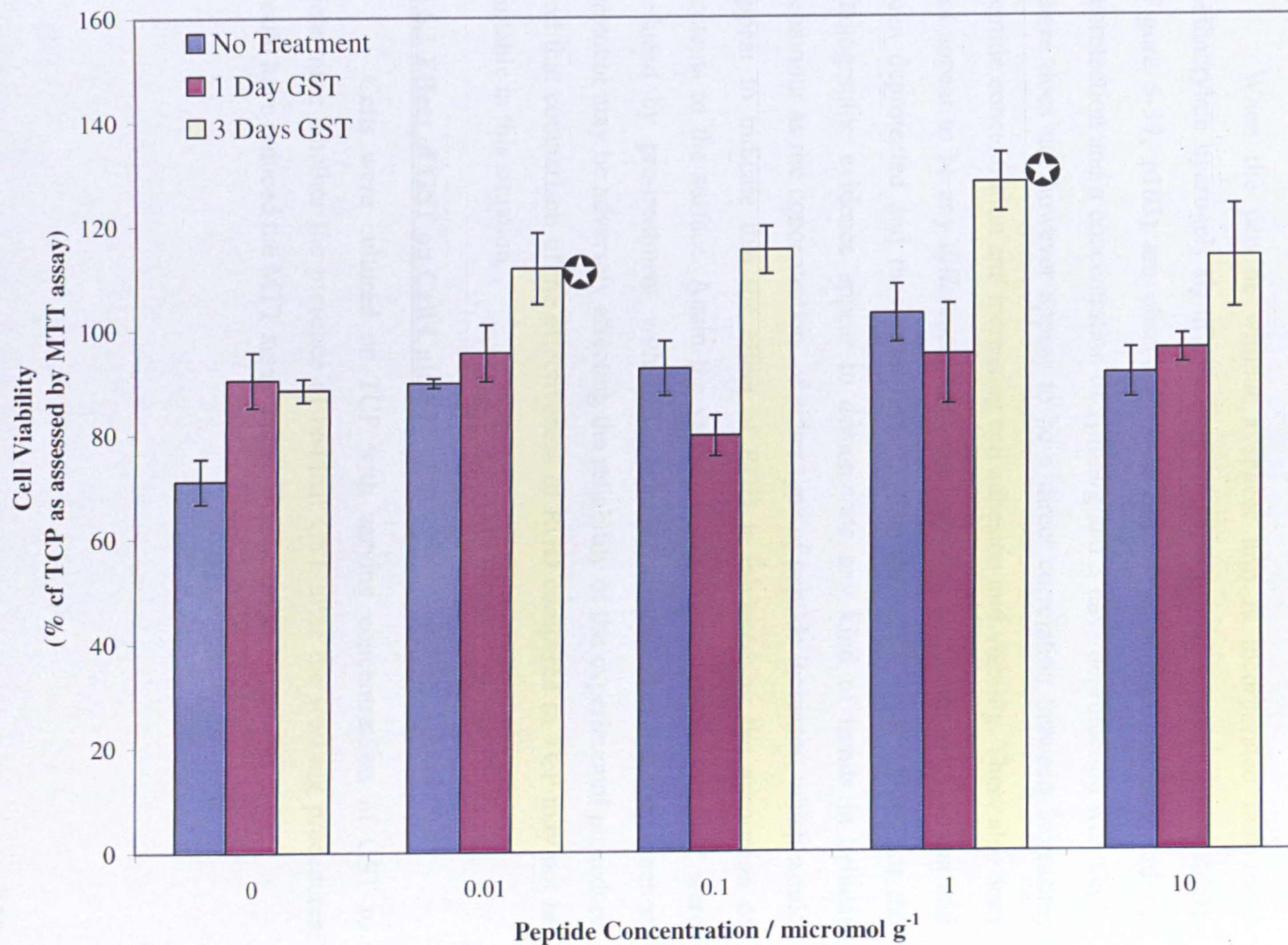


Figure 5-39 Viability of human dermal fibroblasts cultured in media containing 10% FCS for 24 hours on butyl methacrylate polymers containing varying concentrations of MacGR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates. Polymers were deprotected with 0.05mg/ml GST and 500 μmol dm^{-3} GSH in PBS and 0.5ml per sample for 0, 1 or 3 days (deprotection solution was replaced daily). Viability of cells (F1833, passage 9) was assessed via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the non-peptide containing control that has been treated with the same amount of GST (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

The MTT assay results for the viability of cells cultured on butyl methacrylate hydrogels containing the long peptide sequence MacAddaGR(4-Bbs)GDS (Figure 5-38, p103) show no trends in increasing cell number and viability as the peptide concentration increases. However there is a significant decrease in cell number and viability on some of the polymer surfaces that have been pre-treated with GST for 3 days (0.1 and 1μmol/g). However this reduction in cell adhesion and viability appears to be partly due to the adsorption of GST to the surfaces as the average reading given for the MTT assay of cells on TCP also decreased when it had been pre-treated with GST.

When the peptide without a spacer arm is incorporated into butyl methacrylate hydrogels significant improvements in cell adhesion and viability (Figure 5-39, p103) are observed for a concentration of 0.1μmol/g and no deprotection and a concentration of 1μmol/g and 3 days deprotection with GST. There does not however appear to be a direct correlation between increasing peptide concentration and increasing cell adhesion and viability. There also does not appear to be any differences between polymers containing peptide that had been deprotected and those that had. Neither the MTT assay values nor the photographic evidence appear to demonstrate any kind of trends in cellular behaviour as the concentration of either type of peptide increases, which would appear to indicate that the effect of RGD is inhibited by the adsorption of proteins to the surface. Again the values of MTT assay results for TCP were reduced by pre-treatment with GST, which indicated that the deprotection protocol may be adversely affecting the reliability of the experimental procedure and that comparison of the effectiveness of RGD compared to TCP may not be suitable in this situation.

5.6.3 Effect of GST on Cell Culture

Cells were cultured on TCP with varying concentrations of GST to determine whether the presence of residual GST after the washing procedures could have reduced the MTT assay value.

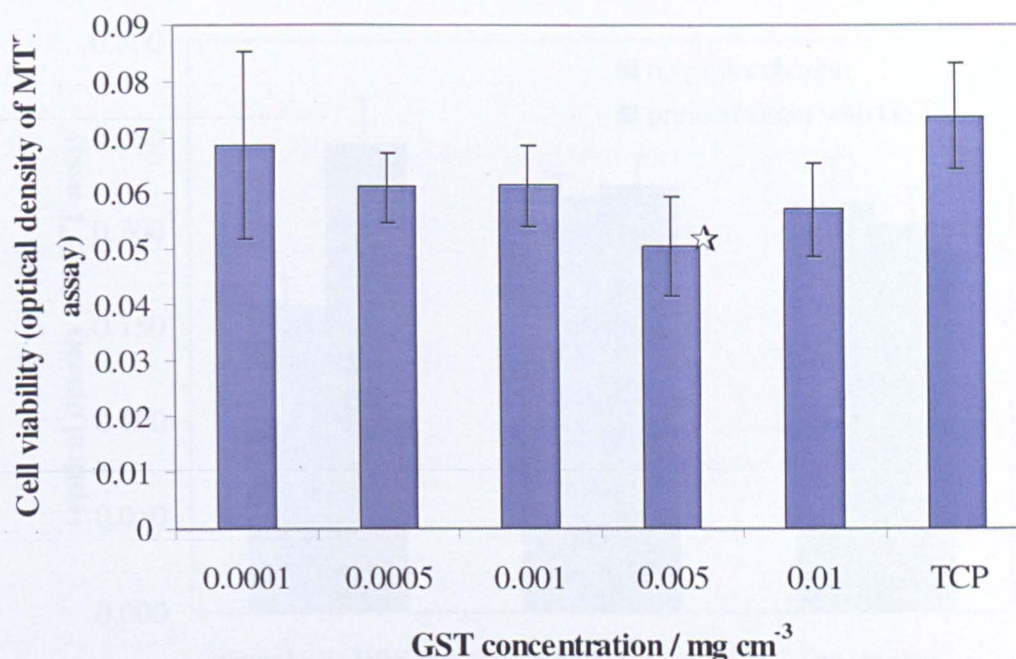


Figure 5-40 Viability of human dermal fibroblasts cultured in media containing 10% FCS for 24 hours on tissue culture plastic containing varying concentrations of GST/GSH in the media. Viability of cells (F1833, passage 8) was assessed via the MTT assay and significance was analysed via the Student's non-paired T-test compared to no additive in the media (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

The MTT results show that there is a slight decrease in recorded MTT values as the concentration of GST present in the media increases. This may be due to a reduction in cell number caused by competitive protein adsorption or a reduction in cell activity caused by interaction of the GST with the cells. A significant reduction in viability was found at GST concentrations of 0.005mg/ml compared to the deprotection solution of 0.05mg/ml. In order for a significant effect to be seen from the GST deprotection protocol over 10% of the GST utilised in the deprotection step must have remained on the polymer. This seems unlikely as the polymers were sterilised by washing with ethanol and then washed a further three times with PBS after the deprotection and prior to cell culture. However the addition of ethanol may have caused large amounts of enzyme to precipitate out and form a coating of the enzyme on the surface thus preventing interaction of the peptide with the cells. The effect of the deprotection protocols and washing procedures on TCP was examined.

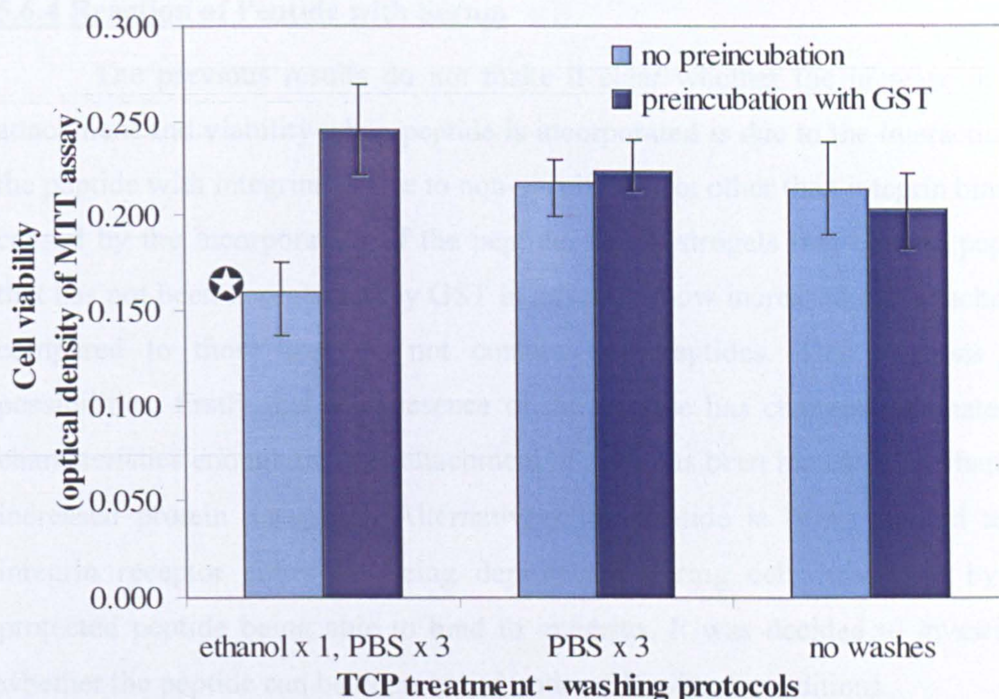


Figure 5-41 Viability of human dermal fibroblasts cultured for 24 hours in media containing 10% FCS on tissue culture plastic after GST treatment and various washing procedures. Viability of cells (F1833, passage 8) was assessed via the MTT assay and significance was analysed via the Student's non-paired T-test compared to TCP without any treatment. (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

The analysis showed that neither GST treatment or PBS washes caused a significant alteration in the viability of cells grown on TCP. However washing with ethanol and PBS caused a significant reduction in the viability of cells grown on the TCP, which was ameliorated by the GST treatment prior to the ethanol wash. It appears that the ethanol wash may have an adverse effect on the ability of materials to adhere and support viable cells and consequently it was decided to alter the existing protocol and perform the deprotection step after the sterilisation of the polymers.

Polymers were sterilized in ethanol as sheets, washed with PBS to remove traces of ethanol and cut to size in a sterile culture hood before being placed in sterile culture wells. The enzyme deprotection solution was filter sterilized prior to use and the deprotection was conducted in situ with three PBS washes and two serum free media washes prior to cell culture.

5.6.4 Reaction of Peptide with Serum

The previous results do not make it clear whether the increase in cell attachment and viability when peptide is incorporated is due to the interaction of the peptide with integrins or due to non-specific effect other than integrin binding caused by the incorporation of the peptide. The hydrogels that contain peptide that has not been deprotected by GST in advance show increased cell attachment compared to those that do not contain any peptides. This suggests two possibilities, firstly that the presence of the peptide has changed the materials characteristics enough that the attachment of cells has been increased, perhaps by increased protein adsoption. Alternatively the peptide is being utilised as an integrin receptor either by being deprotected during cell culture or by the protected peptide being able to bind to integrins. It was decided to investigate whether the peptide can be deprotected under cell culture conditions.

The short peptide sequence MacGR(4-Bbs)GDS was added in varying concentrations to PBS containing 10% FCS and the GST deprotection solution (0.05mg/ml GST and 500μM GSH in PBS). The absorbance of each reaction solution at 405nm was determined and then the solutions were incubated at 37° C and 5% CO_2. The absorbance of each reaction well was then determined at set time intervals. The change in absorbance values over time was recorded. The differences between the change in absorption of the peptide containing solutions and the non-peptide containing solutions over 24 hours reflect the concentration of the 4-bromobenzyl sulphonamide protecting group in solution.

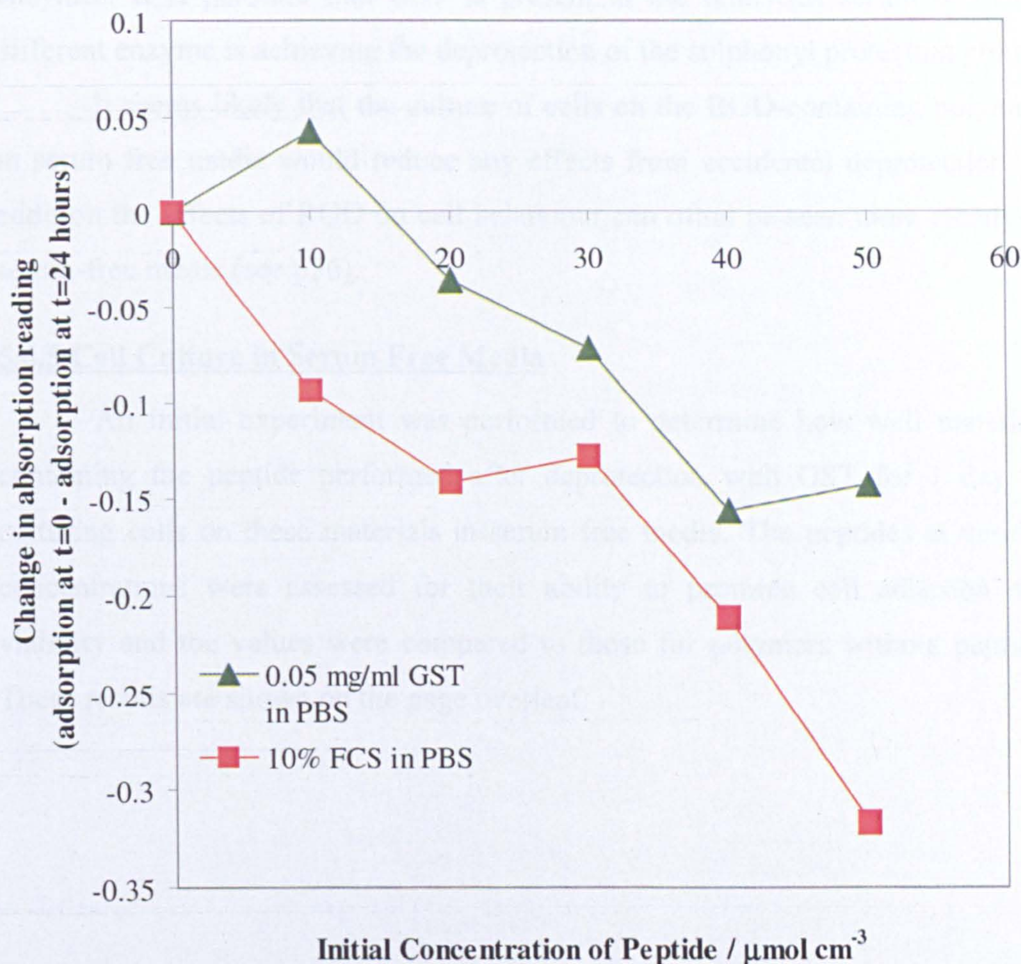


Figure 5-42 Change in Adsorption at 405nm of MacGR(4-Bbs)GDS incubated with various solutions over 24 horus.

The change in adsorption for peptide solutions incubated with GST for 24 hours shows a decrease in optical density proportional to the initial concentration of the peptide. This implies that the peptide has been deprotected and that the amount that has been deprotected is proportional to the initial amount and the reaction has reaching equilibrium. This corroborates the results shown earlier where the deprotection of the arginine derivatives when incubated with GST reached equilibrium as shown in Table 5-4, p103. Adsorption readings for incubations of the peptide in PBS containing 10% fetal calf serum follow a similar trend of decreasing change in optical density as the peptide concentration increases. This implies that the presence of FCS in the media has had the same effect on the peptide as the GST deprotection solution and removed the 4-bromobenzylsulphonyl protecting group. The composition of fetal calf serum has not been fully defined but it is known to contain many different proteins and

enzymes. It is possible that GST is present in the fetal calf serum or that a different enzyme is achieving the deprotection of the sulphonyl protecting group.

It seems likely that the culture of cells on the RGD-containing polymers in serum free media would reduce any effects from accidental deprotection. In addition the effects of RGD on cell behaviour can often be seen more clearly in serum-free media (see p56).

5.6.5 Cell Culture in Serum Free Media

An initial experiment was performed to determine how well materials containing the peptide performed after deprotection with GST for 1 day by culturing cells on these materials in serum free media. The peptides at various concentrations were assessed for their ability to promote cell adhesion and viability and the values were compared to those for polymers without peptide. These results are shown on the page overleaf.

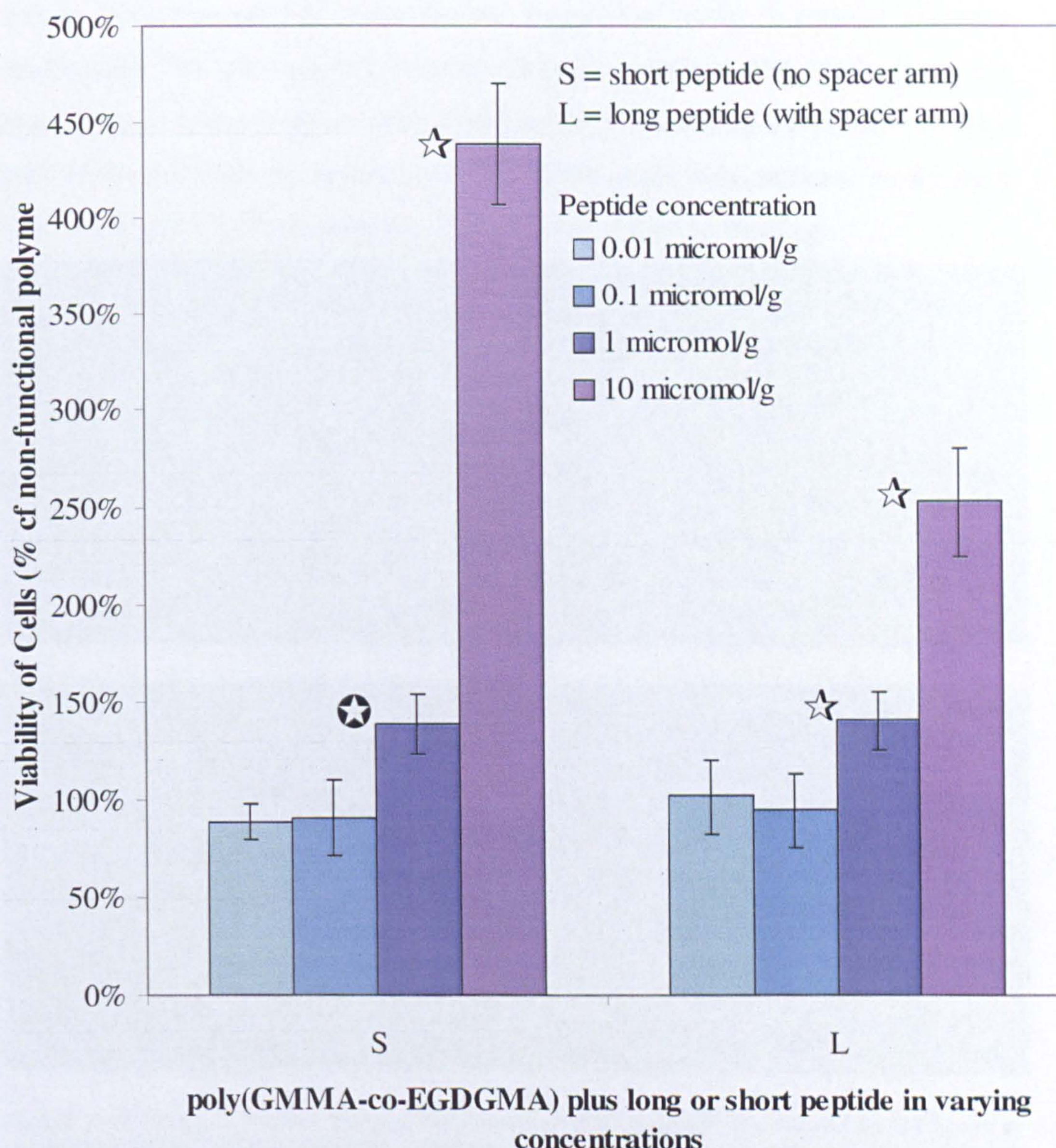


Figure 5-43 Viability of human dermal fibroblasts cultured in serum-free media for 24 hours on glycerol methacrylate hydrogels containing varying concentrations of MacAddaGR(4-Bbs)GDS or MacGR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates with errors shown being standard deviation divided by the number of replicates. Polymers were deprotected with 0.05mg/ml GST and 500µmol dm^{-3} GSH in PBS and 0.5ml per sample for 1 day. Viability of cells (F1833, passage 9) were assessed via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the non-peptide containing control that has been treated with GST for 1 day (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

In the glycerol methacrylate containing polymers the two lowest concentrations of peptide (0.01 and 0.1µmol/g) show no increase in the cell number and viability over the background substrate. At a concentration of 1 µmol/g of either peptide there is a small increase and at the higher concentration of 10 µmol/g of peptide there is a large increase. This indicates that there is a

certain minimum peptide concentration required to evoke a cellular response. Interestingly the short peptide provokes a larger response than the long peptide. In a situation where a spacer arm is not required to distance the peptide from the bulk of the polymer the added flexibility of the chain may decrease its ability to bind to integrins by increasing the entropy loss caused by binding.

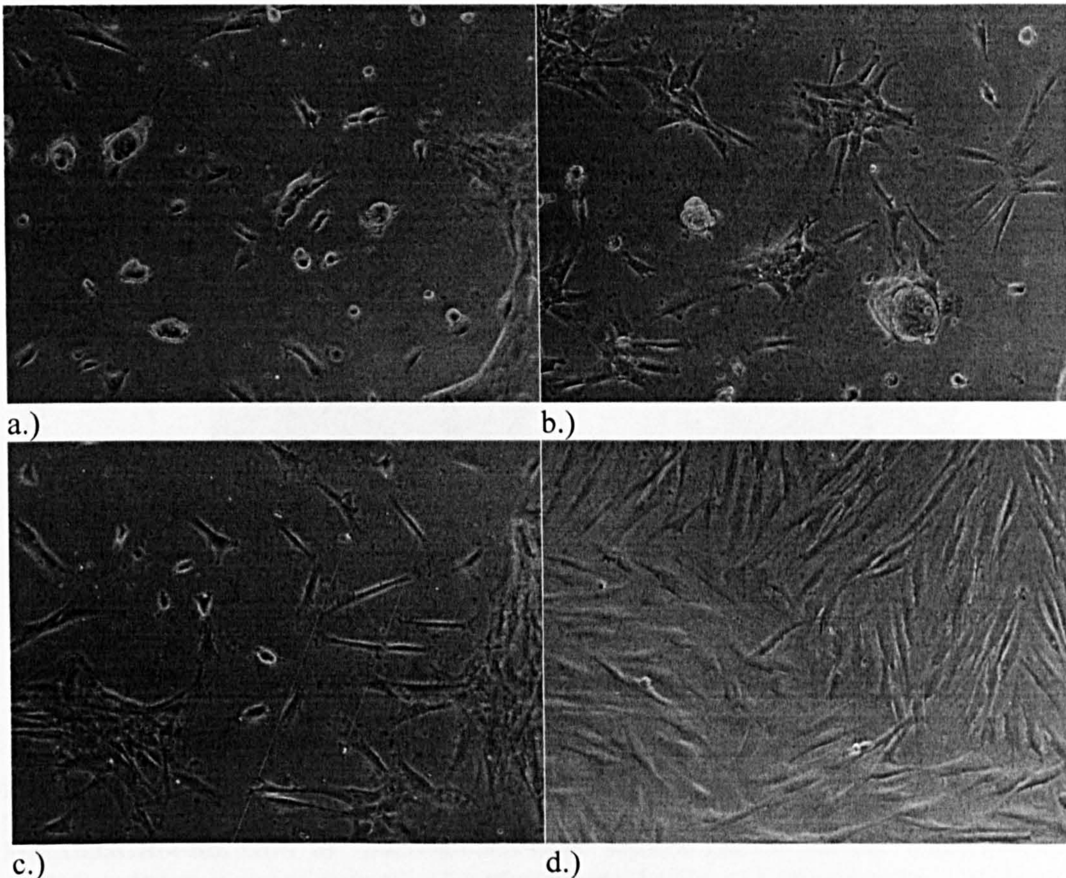


Figure 5-44 Primary human dermal fibroblasts (F1833, passage 9) cultured for 24 hours in serum-free media on poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) pre-incubated with 0.05mg/ml GST and 500 μmol dm^{-3} GSH for 1 day a.) 0.01 μmol/g peptide, b.) 0.1 μmol/g peptide, c.) 1 μmol/g peptide and d.) 10 μmol/g peptide

The photographs of the cells corroborate the trends shown in the MTT assay results (Figure 5.38) where the lower concentrations of peptide give results similar to the background substrate and the higher concentrations of peptide promote cell adhesion and spreading.

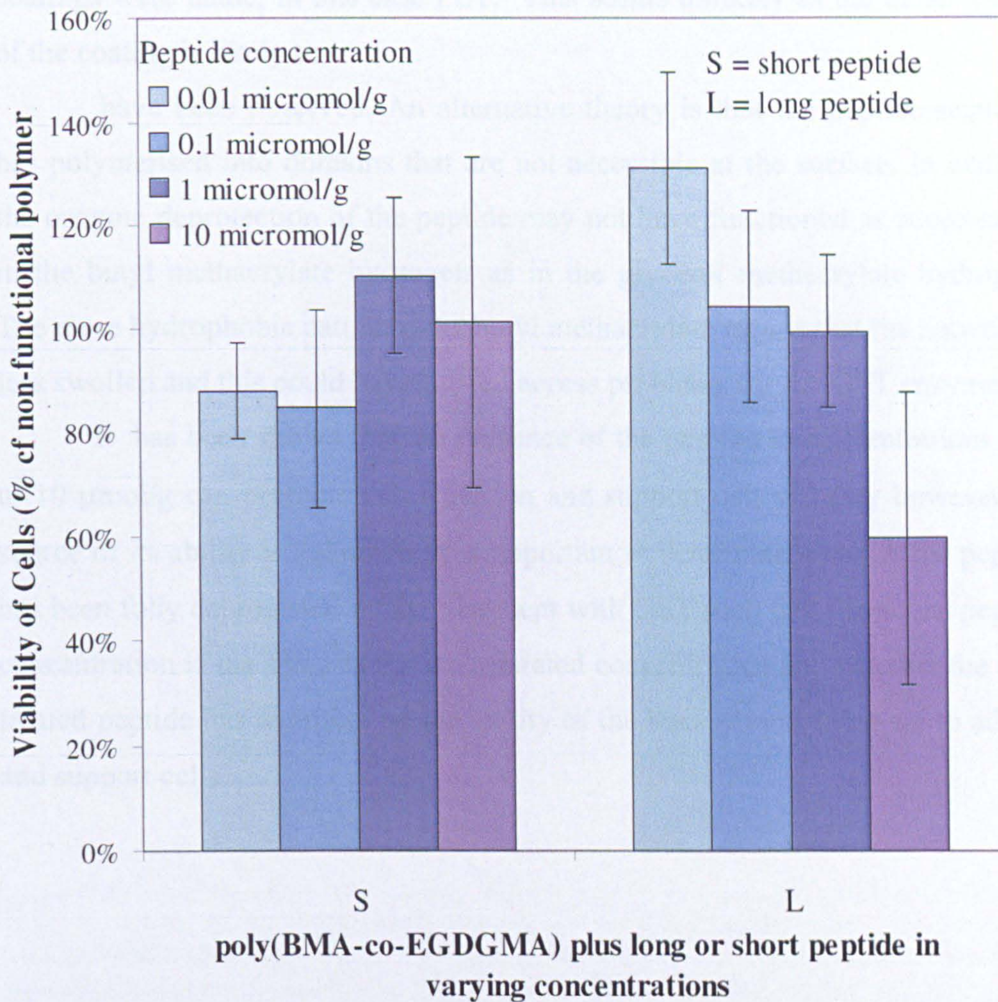


Figure 5-45 Viability of human dermal fibroblasts cultured in serum-free media for 24 hours on butyl methacrylate hydrogels containing varying concentrations of MacAddaGR(4-Bbs)GDS or MacGR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates. Polymers were deprotected with 0.05mg/ml GST and 500 μmol dm^{-3} GSH in PBS and 0.5ml per sample for 1 day. Viability of cells (F1833, passage 9) were assessed via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the non-peptide containing control that has been treated with GST for 1 day (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

The butyl methacrylate based polymers showed no trends in increasing cell attachment and viability as the peptide concentration increased either with the long peptide or the short peptide as shown in Figure 5.39 above. The lack of improvement in the cell adhesion and viability in the butyl methacrylate hydrogels on incorporation of the peptide sequence is likely to be due to the competing effects of protein adsorption but there are alternative explanations. The possibility exists that the polymer coating may have become delaminated and no longer be present and all values refer to the substrate on which the

coatings were made, in this case PET. This seems unlikely as the delamination of the coating is likely to

have been observed. An alternative theory is that the peptide sequence has polymerised into domains that are not accessible at the surface. In addition the enzyme deprotection of the peptide may not have functioned as successfully in the butyl methacrylate hydrogels as in the glycerol methacrylate hydrogels. The more hydrophobic nature of the butyl methacrylate means that the network is less swollen and this could have caused access problems for the GST enzyme.

It has been shown that the presence of the peptide in concentrations of 1 to 10 μmol/g can promote cell adhesion and support cell viability however the source of its ability is unknown. It is important to determine whether the peptide has been fully deprotected by the treatment with GST such that the actual peptide concentration is the same as the incorporated concentration and whether the non-treated peptide has an effect on the ability of the background substrate to adhere and support cells.

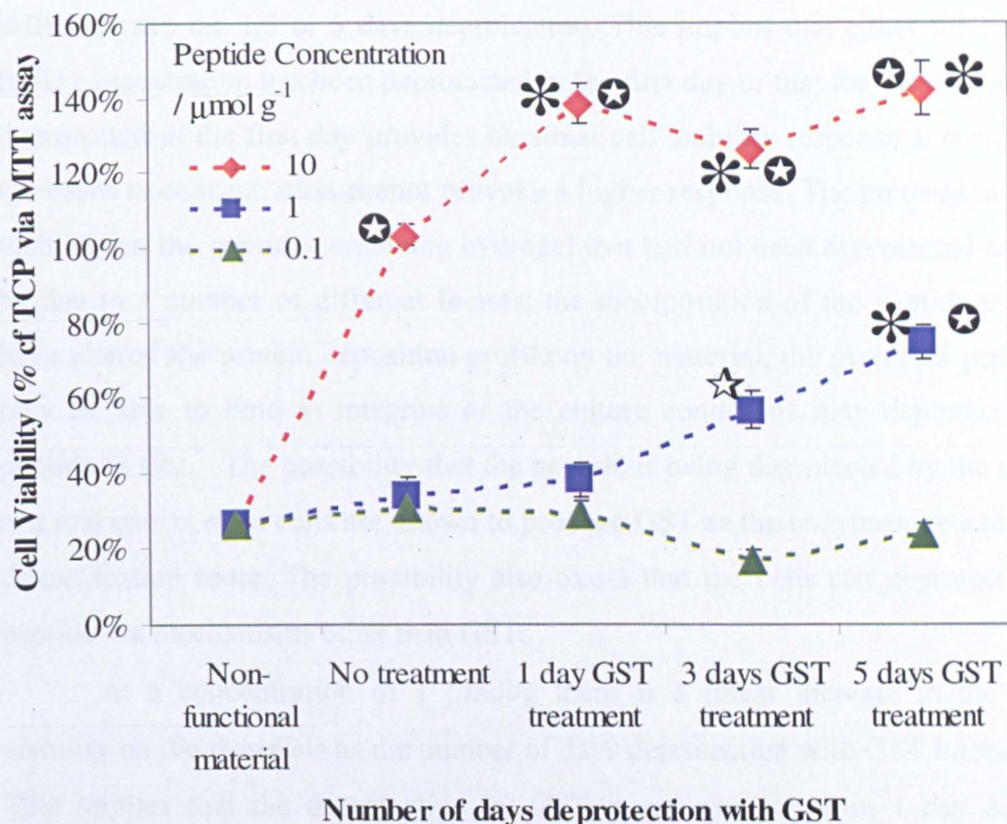


Figure 5-46 Viability of human dermal fibroblasts cultured in serum-free media for 24 hours on glycerol methacrylate polymers containing varying concentrations of Mac-Adda-GR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates and errors show the standard deviation divided by the number of replicates. Polymers were deprotected with 0.05mg/ml GST and 500 GSH µmol dm^{-3} in PBS and 0.5ml per sample for 0, 1, 3 or 5 days (deprotection solution was replaced daily). Viability of cells was assessed (F1833, passage 9) via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the non-peptide containing control that has been treated with the same amount of GST (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$). In addition the significance between 1,3 and 5 days deprotection was analysed compared to the peptide containing polymer that had not been deprotected (* indicates $p<0.05$)

These results show that with the low concentration of RGD at 0.1 µmol/g there is no improvement in cell number and viability as the time of deprotection increases. It is believed that this concentration of peptide is too low to support cell attachment and survival. When the maximum RGD concentration of 10 µmol / g is incorporated in its protected form there is a significant increase in cell viability. The same composition hydrogels that have had the deprotection removed by GST treatment for 1,3 or 5 days also provide a statistically significant increase in cell viability over the non-peptide containing control. The difference between no GST treatment and 1,3 or 5 days is statistically significant with any number of days GST treatment providing an increase in cell viability

however there is no significant change in the ability of the polymer to support cells between the 1,3 or 5 days deprotection. This implies that either the entire RGD concentration has been deprotected in the first day or that the concentration deprotected in the first day provides maximal cell viability response and further increases in concentration cannot provoke a higher response. The increase in cell viability on the peptide-containing hydrogel that had not been deprotected could be due to a number of different factors; the incorporation of the peptide might have altered the protein deposition profile on the material, the protected peptide may be able to bind to integrins or the culture conditions may deprotect the peptide in situ. The possibility that the peptide is being deprotected by the cells is a real one as most cells are known to produce GST as the enzymes are a major detoxification route. The possibility also exists that the cells can deprotect the peptide via mechanisms other than GST.

At a concentration of 1 μmol/g there is a linear increase in the cell viability on the materials as the number of days deprotection with GST increases. This implies that the deprotection by GST is not completed in 1 day and a percentage of the total RGD concentration becomes available for each day that the peptide is treated with GST. This would imply that at the higher concentration there has been a maximal cell response to the RGD concentration after 1 days treatment with GST even though the concentration of RGD available increases on a percentage per day scale.

The linear nature of the increase in cell response to number of days deprotection allows for some speculation as to the percentage of peptide deprotected per day. If the maximal cell response is set to 140% of the TCP control the line for the increased cell response per days deprotection for the 1 mmol/g substrate would intersect it after approximately 13 days. If the 1 μmol/g had been 98% deprotected after this time then there would be ~30% deprotection of the peptide remaining each day per day. However it is unlikely that the linear response of the cells to the number of days deprotection will continue as it has not been shown that cellular response to increasing RGD concentration is linear. In addition it is not known whether the peptide would be fully deprotected after 13 days deprotection. If there was a 30% deprotection of peptide per days treatment with GST then the polymer containing 10μmol/g of peptide would

have an active concentration of 3 μmol/g after 1 day, which appears to be sufficient to promote maximal cell adhesion and viability.

It is apparent that the equilibrium position of the deprotection reaction has not been pushed towards complete deprotection by the heterogenous nature of the reaction between soluble GST and the immobilised peptide. It appears likely that the extent of deprotection is equal to or less than 30%. Consequently it becomes necessary to analyse the exact amount of peptide that is being deprotected each day by GST and hence is available to cells. This required the synthesis of bulk modified polymers that contain enough mass to be analysed. To determine the actual concentration of peptide that is available towards cells it is also important to determine whether the culture conditions can further increase the amount of accessible peptide by deprotecting RGD moieties during culture.

5.6.6 Peptide Concentration Analysis

Hydrogel sheets containing 5 or 0.5μmol/g of MacGR(4-Bbs)GDS were synthesised by UV polymerisation of sheets. The concentration of peptide was reduced from the 10 or 1μmol/g utilised in previous cell culture experiments to allow a greater mass of hydrogels to be synthesised thus enabling a greater number of different analyses to be carried out. As this polymerisation method does not allow the fabrication of butyl methacrylate polymers then only glycerol methacrylate hydrogels were created. Hydrogel samples were incubated with PBS, 500μM GSH in PBS or 0.05mg/ml GST and 500μM GSH for 3 or 5 days. These experiments were also performed on the hydrogel that did not contain peptide. The hydrogels were washed with PBS and then distilled water. The samples were then weighed and the equilibrium water content (EWC) was calculated.

$$EWC = \frac{\left(wet\ weight - dry\ \ weight\right)}{wet\ weight}$$

This is important as the amount of peptide perceived by cells will be affected by the swelling of the hydrogels as the amount of peptide present will be diluted over a larger volume as the hydrogel swells.

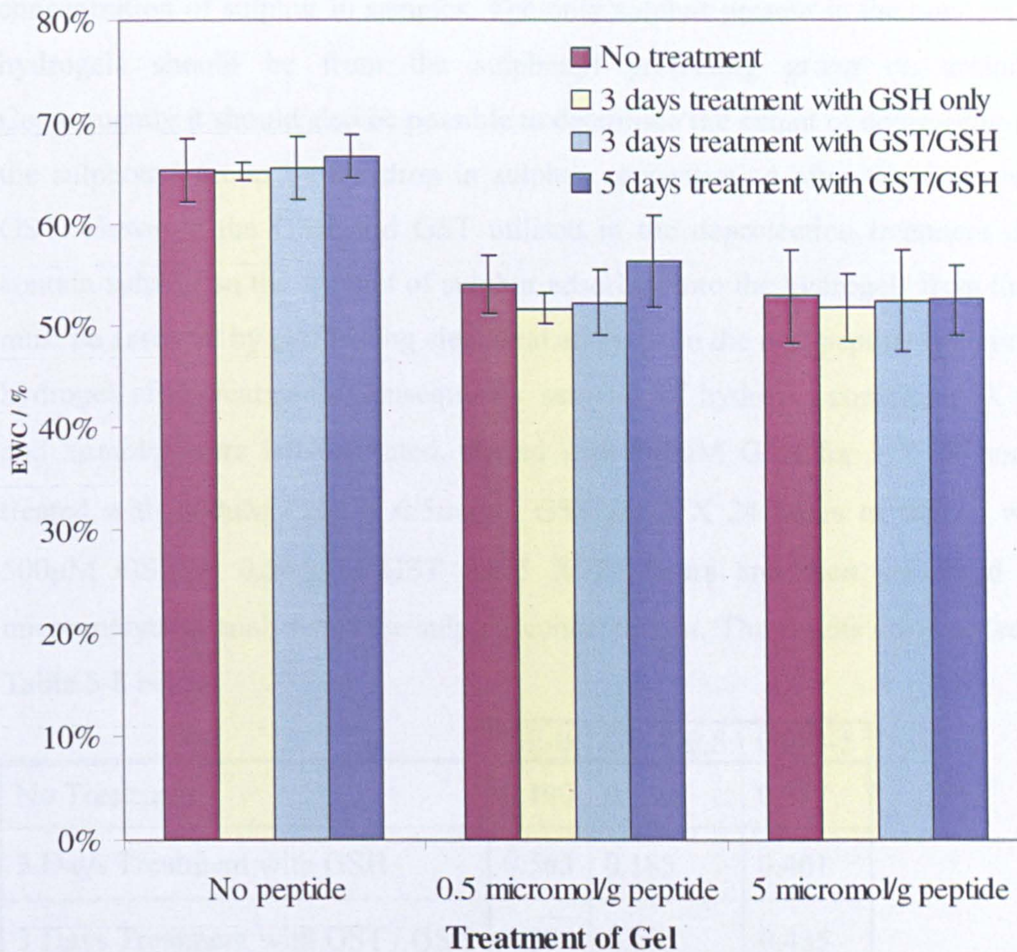


Figure 5-47 Equilibrium water content of poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) hydrogels in distilled water after incubation with PBS, GSH or GSH/GST. Results are the mean of 3 replicates and the errors shown are the standard deviation.

The equilibrium water content of the polymers did not change when the polymers were pre-treated with GSH or GST/GSH. However the equilibrium water contents of the peptide-containing polymers were lower than the EWC for the non-peptide containing control hydrogels. The amount of water present in the hydrogels does not change when the concentration of the peptide changes so it is not that the peptide that is adsorbing less water than the bulk of the hydrogels. It seems likely that the presence of the peptide has affected the polymerisation rates of the monomers and consequently a less porous and less expandable network has been formed that is not capable of adsorbing as much water as the blank hydrogel.

After EWC measurements were taken samples were then dried and then sent for elemental analysis. It should be possible to determine the amount of peptide present in the hydrogels by use of microanalytical techniques to find the

concentration of sulphur in samples. The only sulphur present in the non-treated hydrogels should be from the sulphonyl protecting group on arginine. Consequently it should also be possible to determine the extent of deprotection of the sulphonyl group by the drop in sulphur concentration after treatment with GST. However the GSH and GST utilised in the deprotection treatment also contain sulphur so the amount of sulphur adsorbed into the hydrogels from these must be assessed by performing elemental analysis on the non-peptide containing hydrogel after treatment. Consequently samples of hydrogel containing 0, 0.5 and 5μmol/g were left untreated, treated with 500μM GSH for 3 X 24 hours, treated with 500μM GSH + 0.5mg/ml GST for 3 X 24 hours or treated with 500μM GSH + 0.5mg/ml GST for 5 X 24 hours and then submitted for microanalytical analysis of the sulphur concentration. The results are detailed in Table 5-8 below.

	GE-0	GE-S-0.5	GE-S-5
No Treatment	0.190	0.130	0.377
3 Days Treatment with GSH	0.563	0.185	0.401
3 Days Treatment with GST / GSH	0.874	0.185	0.435
5 Days Treatment with GST / GSH	1.060	0.403	0.487

Table 5-8: Elemental Analysis for Concentration of Sulphur in Glycerol Methacrylate Hydrogels containing 0, 0.5 or 5μmol/g of MacGR(4-Bbs)GDS. Hydrogels were washed with PBS, treated with 500 μmol dm^{-3} GSH for 3 X 24 hours, treated with 500μM GSH + 0.5mg/ml GST for 3 X 24 hours or treated with 500μM GSH + 0.5mg/ml GST for 5 X 24 hours. Hydrogels were then washed X 3 with PBS, X 5 with deionised H_2O and then X 5 with ethanol prior to drying and analysis.

From the sulphur concentrations determined for the non-peptide containing hydrogel (GE-0) it can be seen that there is a fairly large concentration of sulphur in the non-treated sample. It is not known what the source of this sulphur was as the polymer should not have been exposed to sulphur at any point in its treatment as the water utilised for washing procedures was deionised. It is possible that the polymer has adsorbed atmospheric sulphur. The non-peptide containing hydrogel adsorbs and retains large amounts of sulphur used in the deprotection treatment of the treatment. More sulphur is found in the hydrogels after 5 days treatment with GST/GSH than after 3 days treatment with GST/GSH indicating that GST and GSH adsorb strongly to the polymer and are not removed by the washing procedures.

The amount of sulphur found in the polymer containing a nominal peptide concentration of 0.5μmol/g was less than that found in the non-peptide containing polymer. In addition the concentration of sulphur found was only 0.130μmol/g that, if it was only due to sulphur from the peptide, would correspond to only 25% incorporation of the peptide. It seems likely that at least some of the found sulphur concentration will be due to the same source as provided the sulphur concentration in the non-peptide containing hydrogel. The amount of sulphur in the hydrogels containing a nominal 0.5μmol/g of peptide increased after treatment with GSH or GST/GSH but the increases were less than that found for the non-peptide containing hydrogel.

The amount of sulphur found in the polymer containing a nominal peptide concentration of 5μmol/g was found to be 0.377μmol/g. If this concentration was only due to sulphur from the peptide it would correspond to 7.5% incorporation of the peptide. It seems likely that at least some of the found sulphur concentration will be due to the same source as provided the sulphur concentration in the non-peptide containing hydrogel. Deducting the amount of sulphur in the non-treated non-peptide containing hydrogel from the amount of sulphur in the nominal 5μmol/g of peptide hydrogel gives a sulphur concentration due to the peptide of 0.187μmol/g – an incorporation efficiency of 3.7%.

The amount of sulphur in the hydrogels containing nominal peptide concentrations of 0.5 or 5 μmol/g increased after treatment with GSH or GST/GSH but the increases were less than that found for the non-peptide containing hydrogel. It seems likely that the peptide has been partially deprotected but the decrease in sulphur concentration corresponding to the deprotection has not been observed due to the adsorption of GST and GSH. It does not appear as though the adsorption of GST and GSH occurs to the same extent in the peptide containing hydrogels as it does in the non-peptide containing hydrogel. This is probably linked to equilibrium water content, which is much higher for the non-peptide containing hydrogel than for either of the peptide containing hydrogels. It seems as though the presence of the peptide in the polymerisation mixture results in a different rate of polymerisation that leads to a less open structure for the peptide containing hydrogels. These more compact hydrogels would be less able to adsorb water and hence the equilibrium

water content would be less and the less porous structure would mean that GST and GSH would be less able to penetrate into the bulk and hence the concentration of sulphur after treatment would be less.

The amount of sulphur in the polymers with a nominal concentration of 0.5μmol/g is the same where the treatment had been 3 x 24 hours with GSH and 3 X 24 hours with GST and GSH. The non-peptide containing polymers adsorb more sulphur when GST has been used than when just GSH has been used. Consequently it appears as if there has been some loss of sulphur from peptide deprotection when the polymer was treated with GST and GSH that is obscured by the rise in sulphur concentration from the adsorption of GST.

As it was not possible to determine the peptide concentration from the elemental analysis of the polymers it was decided to perform amino acid analysis (AAA). The AAA will not be able to determine the amounts of the protected and deprotected arginine derivatives but will give a measure of what percentage of the peptide in the monomer feed was incorporated into the polymer. The amino acid analysis (performed by Alta Bioscience, Birmingham University) showed immobilised peptide concentrations of 0.16μmol/g and 0.68μmol/g for nominal input concentrations of 0.5 and 5μmol/g respectively. This corresponds to 32.0% and 13.6% incorporation of the peptide into the cross-linked hydrogel.

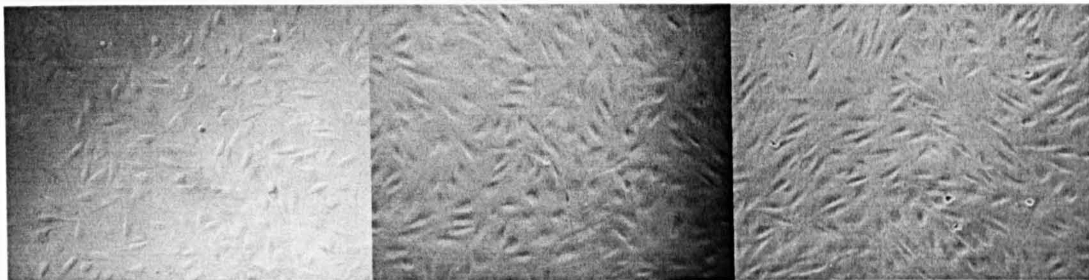
The high EWC of the polymers means that the peptide is spread over a larger volume and the actual peptide concentration perceived by cells is even lower. At the average EWC of 53% for the peptide containing hydrogels the swollen hydrogel contains 47% by volume of the nominal peptide concentration assuming that the polymer has a density of $1 g/cm^3$. This gives actual peptide concentrations of 0.0752 and 0.3196 $\mu mol/cm^3$ for nominal concentrations of 0.5 and 5 μmol/g respectively. The deprotection efficiency was estimated to be ~30% per day, which would correspond to active peptide concentrations of 0.022 and 0.096 $\mu mol/cm^3$ for nominal concentrations of 0.5 and 5 μmol/g respectively .Assuming an integrin penetration depth of 10nm this corresponds to surface peptide concentrations of 0.022 and 0.096 $pmol/cm^2$. Overall the amount of peptide perceived by cells can vary from 3% to 5% of the nominal peptide concentration depending on the input concentration and the deprotection efficiency.

The data shows that peptide incorporation efficiency is dependent on the nominal input concentration and it is difficult to determine the exact amount of peptide incorporation at different input concentrations. Consequently nominal peptide concentrations will be used throughout the remainder of the discussion.

5.6.7 Competition with Soluble RGD

To determine whether the attachment of the cells to the substrates containing peptide was due to the binding of RGD to integrins it was decided to challenge the adhered cells with soluble peptide.

Cells were cultured on TCP and polymers containing varying concentration of MacGR(4-Bbs)GDS for 24 hours in serum free media after which time the media was removed and replaced with media containing 1 mg/ml of RGDS. Photographs were taken at 15 minute intervals up to 60 minutes after the addition of the soluble peptide.



a.) b.) c.)

Figure 5-48 Human dermal fibroblasts cultured in serum-free media on TCP for 24 hours a.) prior to addition of soluble RGDS, b.) 30 mins after addition of soluble RGDS and c.) 60 mins after the addition of soluble RGDS.

There was no change in the morphology or apparent confluency of the cells on TCP after the addition of soluble RGDS. This shows that the addition of the soluble peptide does not affect cells that adhere via mechanisms other than integrin binding. The photographs in Figure 5-49, p103 show the detachment of cells from glycerol methacrylate hydrogels containing 10μmol/g of peptide that had been pretreated with GST for 3 days.

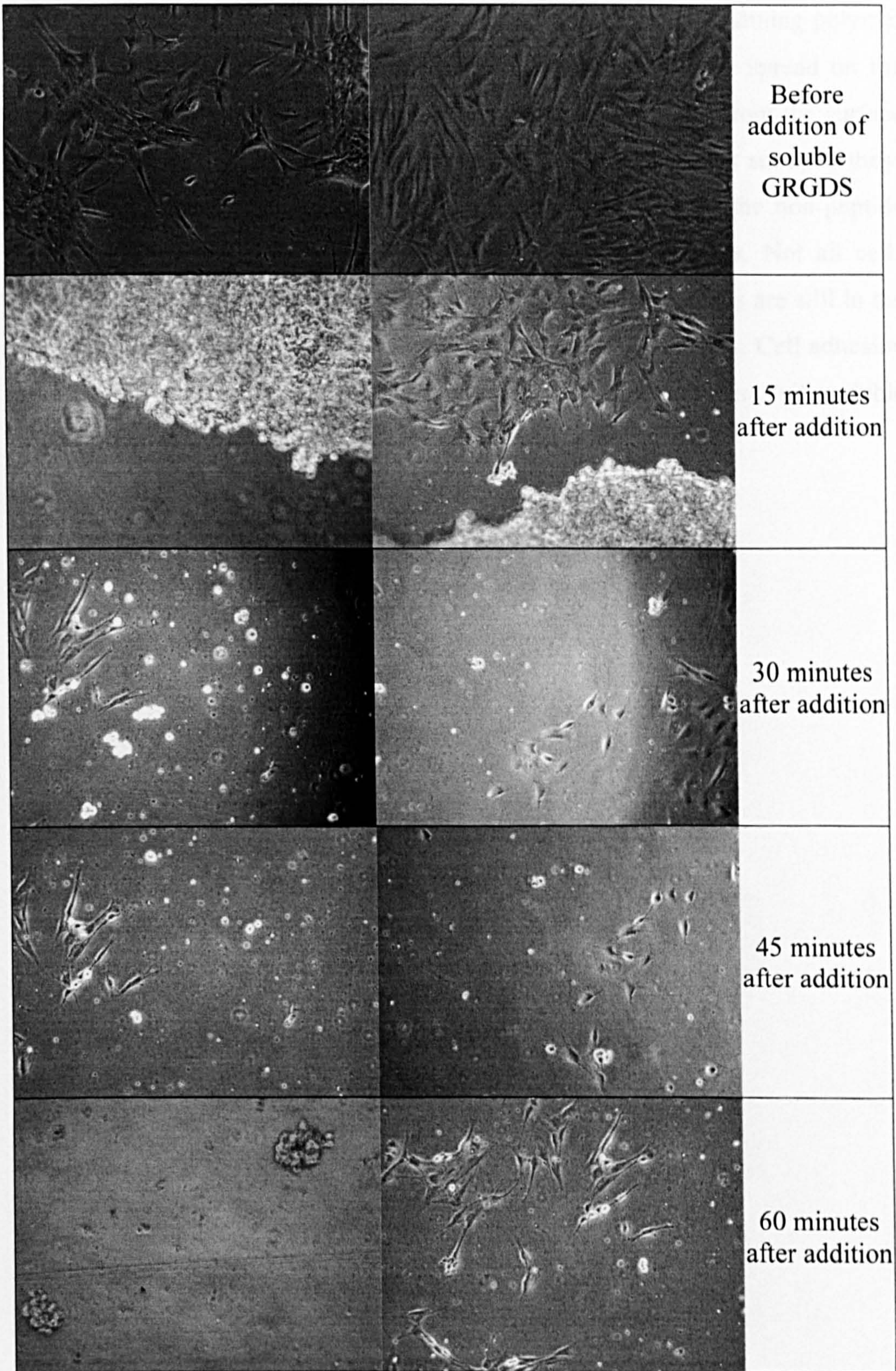


Figure 5-49 Detachment of human dermal fibropblasts (cultured for 24hours in serum free media on GE-S-10-3 at set time points after the addition of 1 mg /ml RGDS in serum free media (2 photographs at each time point)

The photographs show that 15 minutes after the addition of RGDS large sheets of cells have detached from the surface of the peptide-containing polymer but that significant numbers of cells remain attached and fully spread on the surface. Later time points show these cells gradually detaching from the surface and illustrate cells at all stages of detachment. After 60 minutes some of these lifting cells have formed aggregations similar to those seen on the non-peptide containing glycerol methacrylate polymers in other experiments. Not all cells have detached after 60 minutes however it appears that these cells are still in the process of detachment which seems to be gradual and continuous. Cell adhesion and viability results from the MTT assay before and after treatment with soluble RGD are shown on the page overleaf.

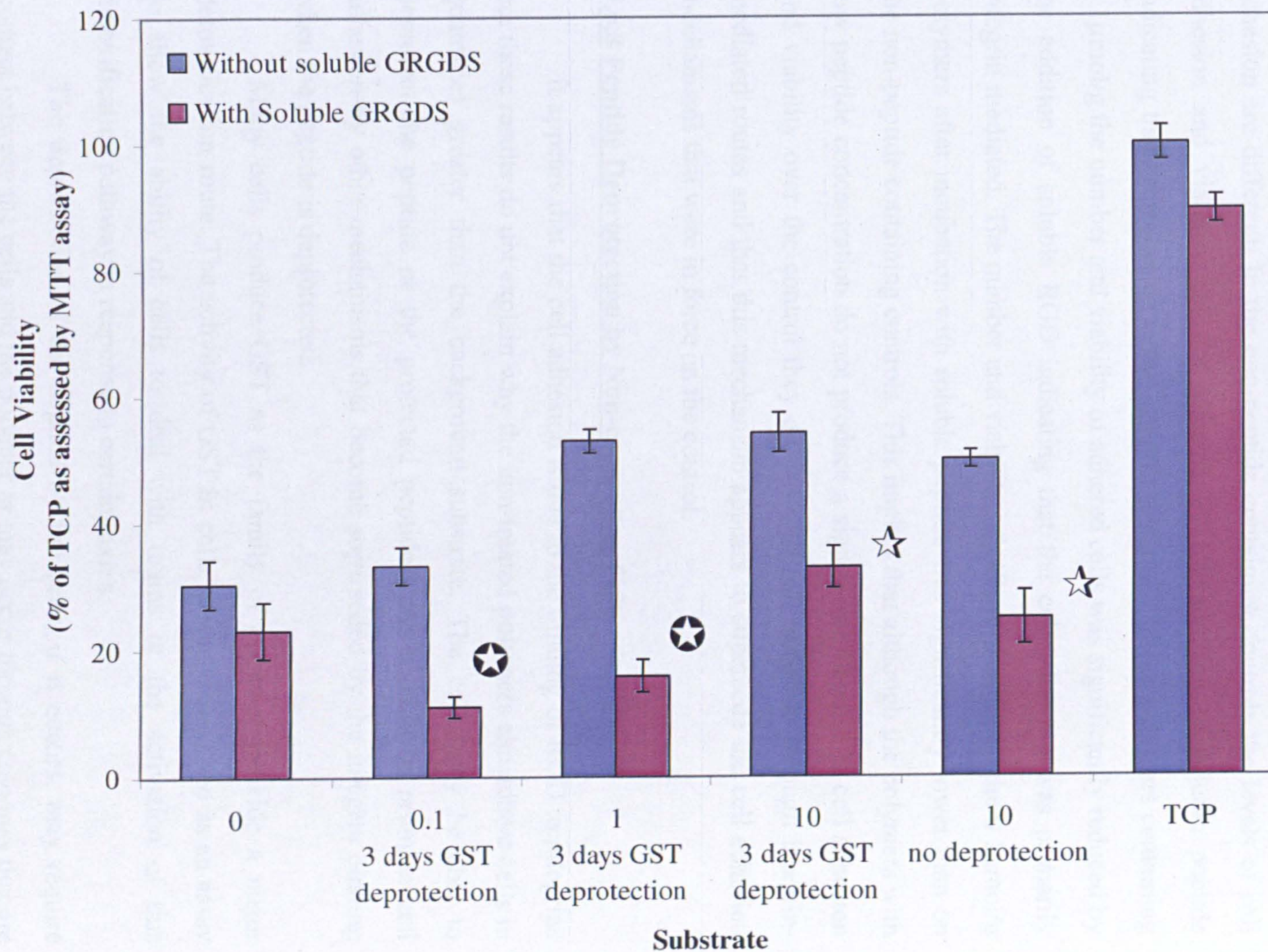


Figure 5-50 Viability of human dermal fibroblasts cultured in serum-free media for 24 hours on glycerol methacrylate polymers containing varying concentrations of MacGR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates and errors show the standard deviation divided by the number of replicates. Polymers were deprotected with 0.05mg/ml GST and 500 μmol dm^{-3} GSH in PBS and 0.5ml per sample for 3 days (deprotection solution was replaced daily). After 24 hours culture the media was removed from all cells and replaced with serum-free media containing 1mg/ml GRGDS or plain serum-free media for 1 hour. Viability of cells was assessed (F002, passage 6) via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the non-peptide containing control (✪ indicates p<0.01 and ☆ indicates p<0.05).

The MTT assay results confirm the visual evidence that cells on peptide containing hydrogels are detached by the addition of soluble GRGDS. In all cases the number and viability of cells on the peptide containing hydrogels were significantly reduced to the level of the non-peptide containing control or below. Interestingly this was also the case on hydrogels containing 0.1 μmol/g of peptide where no significant increase in cell adhesion had been previously reported. It appears as if the same levels of cell adhesion are supported by polymers without peptide and with the low peptide concentration but that the mechanisms of cell adhesion are different. In the non-peptide containing controls the levels of cell adhesion and viability are not affected by the addition of soluble peptide indicating that the adhesion is not integrin mediated. In the polymers containing 0.1 μmol/g the number and viability of adhered cells was significantly reduced by the addition of soluble RGD indicating that the cell adhesion was primarily integrin mediated. The number and viability of cells remaining on the 0.1 μmol/g polymers after incubation with soluble peptide was significantly lower than on the non-peptide containing controls. This implies that although the polymers with low peptide concentration do not produce a significant increase in cell adhesion and viability over the control they do promote cell adhesion through integrin-mediated routes and that this mechanism appears to supersede the cell adhesion mechanisms that were in force on the control.

5.6.8 Peptide Deprotection by Non-contacting Cells

It appears that the cell adhesion is due to the binding of RGD to integrins but these results do not explain why the non-treated polymers can adhere cells in quantities greater than the background substrate. The cells may be able to deprotect the peptide or the protected peptide may be able to promote cell adhesion by other mechanisms that become superseded by the integrin binding when the peptide is deprotected.

Many cells produce GST as the family of enzymes provide a major detoxification route. The activity of GST in cells is sometimes used as an assay to show the ability of cells to deal with toxins or the activation of this detoxification pathway in response to certain toxins.

The deprotection of the peptide by the cells, if it occurs, may require contact between the cells and the polymer or may occur through enzymes that are

secreted by cells and are active in the media. To determine whether the cells are able to deprotect the peptide without contact the polymers were placed at the bottom of wells and cells were placed in co-culture baskets above the polymers. The cell adhesion and viability results from the MTT assay are shown on the page overleaf.

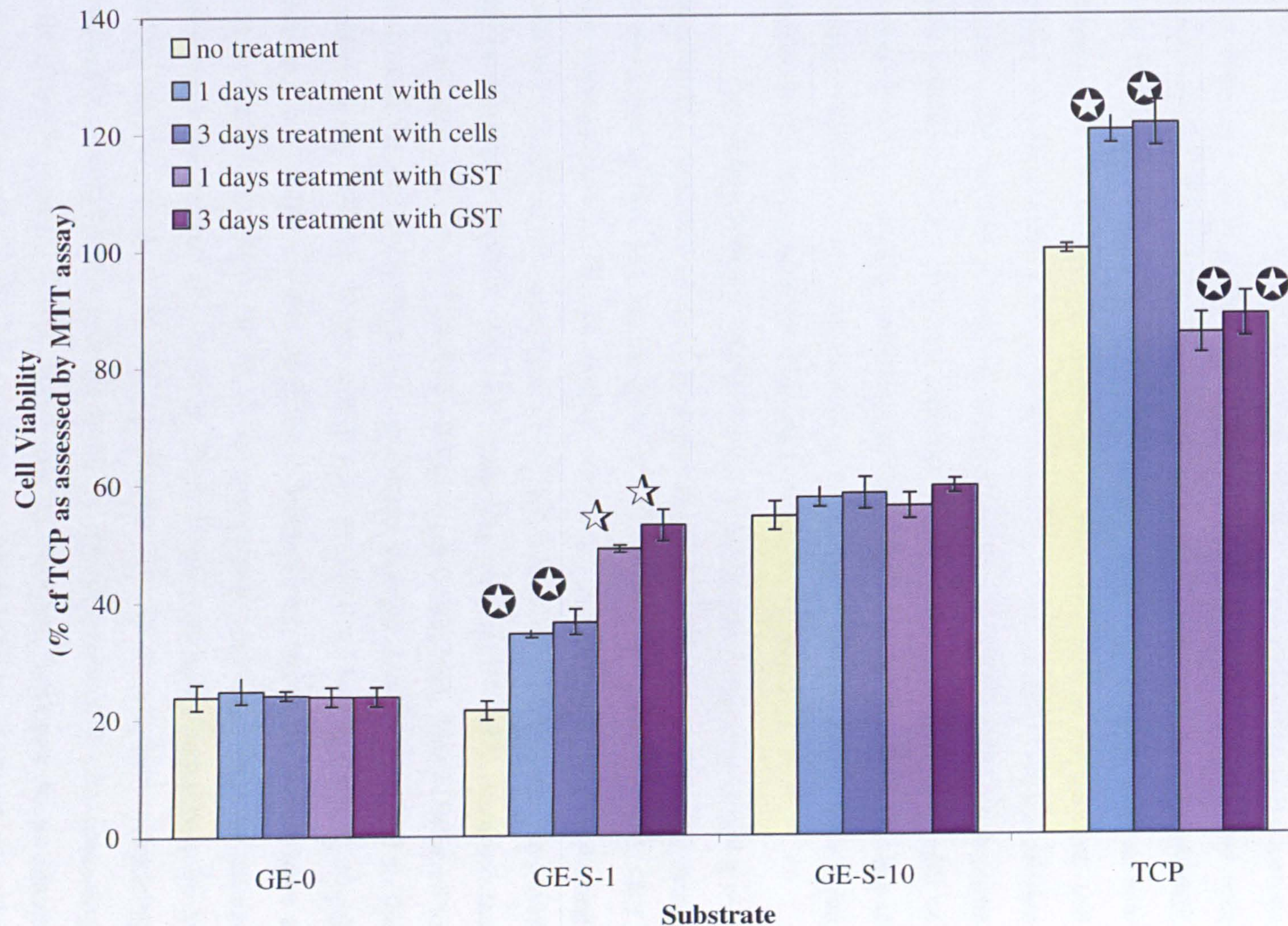


Figure 5-51 Viability of human dermal fibroblasts cultured in serum-free media for 24 hours on glycerol methacrylate polymers containing varying concentrations of MacGR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates and errors show the standard deviation divided by the number of replicates. Polymers were deprotected with 0.05 μmol dm^{-3} GST and 500μM GSH in PBS and 0.5ml per sample or incubated with 50,000 human dermal fibroblasts suspended above the polymers in a co-culture basket for 0, 1 or 3 days (deprotection solution was replaced daily). Viability of cells was assessed (F002, passage 6) via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the peptide-containing control that had not been pre-treated (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

The adhesion and viability of cells on hydrogels containing 10μmol/g of MacGR(4-Bbs)GDS was not significantly affected by pre-treatment with either cells or GST for 1 or 3 days. Previous analysis of hydrogels containing 10μmol/g of this peptide showed a significant increase in cell number and viability when pre-treated with GST (see Figure 5-46, p103). It appears that in this case the non-treated peptide containing polymer promotes maximal cell response that cannot be improved by incubation with either cells or GST. This maximal cell response appears to be ~60% of the TCP control, which is much lower than that observed previously of ~130% of the TCP control. This experiment was conducted with human dermal fibroblasts from a different source as there were not sufficient cells from the previous cell source to continue with. It is possible that this new primary cell source is not as responsive to RGD inclusion as the previous cell source. In addition the passage number may have had an effect on the relative adhesion and viability of cells as it is known that integrin expression declines with passage number. However this experiment was carried out with cells of passage number 6 whereas the previous experiment was carried out with cells of passage number 9 and consequently the reverse situation would have been predicted with this experiment showing higher cell responses c.f. TCP.

The adhesion and viability of cells on hydrogels containing 1μmol/g was significantly increased by pre-treatment with non-contacting cells but the extent of the improvement was not as great as that gained from treatment with GST. The pre-treatment of the non-peptide containing polymer with non-contacting cells did not affect the viability of cells later seeded on the polymers. However the pre-treatment of TCP with non-contacting cells significantly increased the numbers and viability of cells later seeded on these surfaces. This is believed to be due to the non-contacting cells producing proteins that are adsorbed to the surface of the TCP but do not adsorb onto the glycerol methacrylate hydrogel surface. The peptide-containing glycerol methacrylate hydrogels should have a similar protein adsorption profile to the non-peptide containing controls but the presence of the peptide may have an effect on the ability of charged proteins to adsorb onto the surface. As the adhesion of cells to the polymers containing 10μmol/g of peptide was not improved by pre-treatment with non-contacting cells it can be assumed that all the glycerol methacrylate hydrogels do not adsorb proteins regardless of whether a peptide is present. This would imply that the

increase in cell adhesion and viability to the polymers containing 1μmol/g of peptide that had be pre-treated with non-contacting cells was due to the deprotection of the peptide by soluble cell products such as GST.

1.1.2 Passage Number

The results from the previous experiment on the deprotection of the peptide by non-contacting cells were surprising as the relative numbers and viability of cells adhered to the peptide containing hydrogels were much lower in comparison to TCP than previously reported. It was decided to investigate the effect of primary cell passage number on the reported cell number and viability.

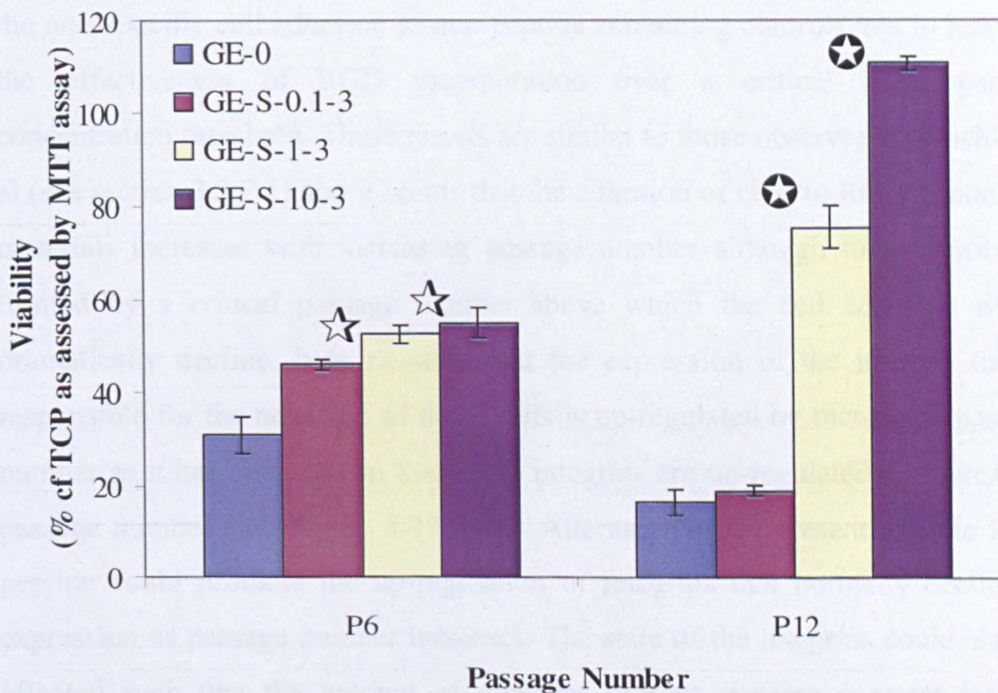


Figure 5-52 Viability of human dermal fibroblasts cultured in serum-free media for 24 hours on glycerol methacrylate polymers containing varying concentrations of MacGR(4-Bbs)GDS. Results shown are the mean of 3-6 replicates and errors show the standard deviation divided by the number of replicates. Polymers were deprotected with 0.05mg/ml GST and 500 μmol dm^{-3} GSH in PBS and 0.5ml per sample for 3 days (deprotection solution was replaced daily). Viability of cells was assessed (F002, passage 6 or passage 12) via the MTT assay and compared to values obtained for TCP. Significance was analysed via the Student's non-paired T-test, each peptide containing polymer was compared to the non-peptide containing control (✪ indicates $p<0.01$ and ☆ indicates $p<0.05$).

At passage number 6 there is a small increase in cell number and viability to hydrogels as the peptide concentration increases. The viability of cells adhered to hydrogels was significantly greater than on the non-peptide containing polymer when the peptide concentration was greater than 1μmol/g. The maximum cell response obtained was ~52% of the TCP control. The viability of

cells adhered to glycerol methacrylate hydrogels without peptide was approximately 30% of the TCP control.

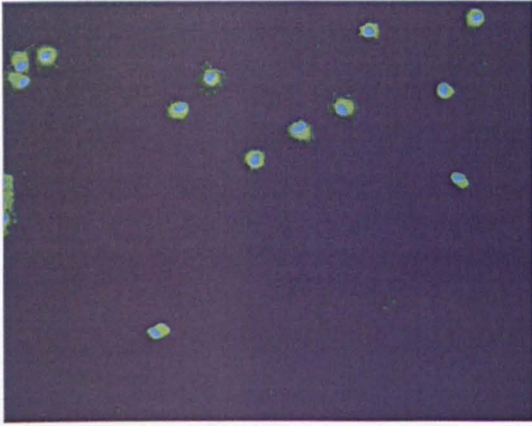
At passage number 12 there is a large increase in cell number and viability to hydrogels as the peptide concentration increases. The viability of cells adhered to the hydrogels is significantly greater than on the non-peptide containing control when the peptide concentration is greater than 1μmol/g. The maximum cell response obtained was ~110% of the TCP control. The viability of cells adhered to glycerol methacrylate hydrogels without peptide was approximately 16% of the TCP control.

The effect of increasing primary cell passage number appears to decrease the non-specific cell adhesion to non-peptide containing controls and to increase the effectiveness of RGD incorporation over a critical RGD peptide concentration threshold. These results are similar to those observed by Jeschke et al (see section 3.3.2.1) and it seems that the adhesion of cells to RGD-containing materials increases with increasing passage number although this is probably limited by a critical passage number above which the cell adhesion would dramatically decline. It is possible that the expression of the integrin that is responsible for the adhesion of these cells is up-regulated by increasing passage number as it has been shown that some integrins are up-regulated by increasing passage number (see Figure 3-13, p54). Alternatively the presence of the RGD peptide could promote the up-regulation of integrins that normally decline in expression as passage number increases. The state of the integrins could also be affected such that the amount of integrins present remains constant but are present in a more active conformation or state.

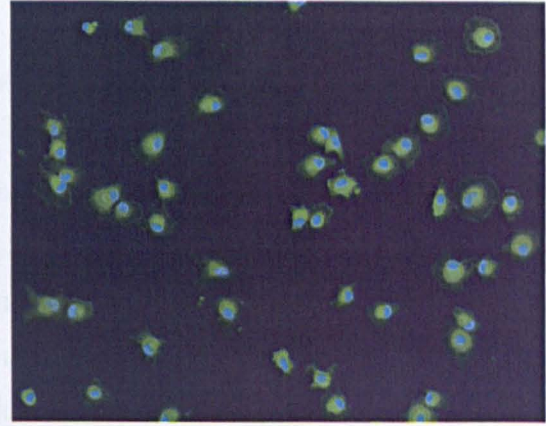
5.6.10 Histology

To investigate whether the presence of the RGD peptide had influenced the amount of cell spreading and F-actin organisation, cells were cultured on hydrogels containing peptide in various concentrations that had been deprotected for 3 days with GST/GSH. Cells were fixed after 30mins, 1, 2, 4 or 24 hours after seeding to investigate the progression of the cell spreading and focal contact formation. The cell nuclei were stained blue with DAPI and F-actin was stained green with FITC-phalloidin.

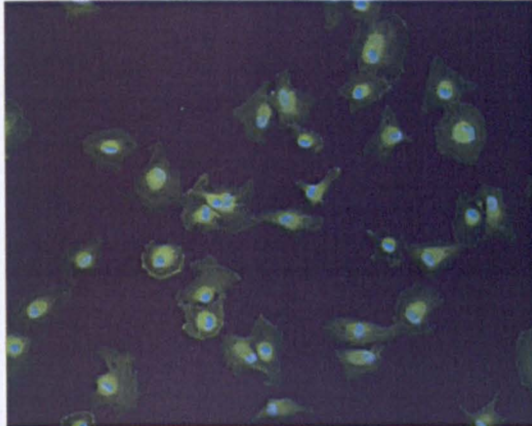
As can be seen in Figure 5-53 (p103) cells adhere and start spreading on TCP within the first 30 minutes after seeding seeding. Spreading increases over time up to 4 hours after seeding and, during this time, the area that the F-actin network around the cells covers increases. At 4 hours after seeding the F-actin network forms an orthogonal shape covering a large area surrounding the cell nucleus and show signs of actin filament formation. At 24 hours after seeding the F-actin has become highly organised and shows highly regulated f-actin fibres within the cell and long fillipodia extending out from the cell.



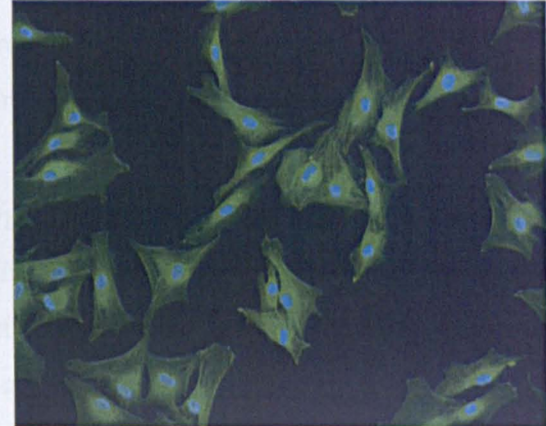
A



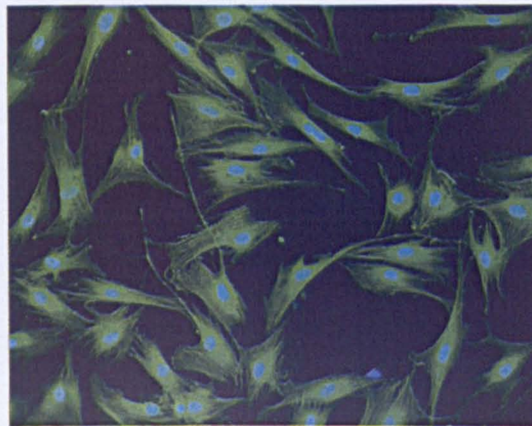
B



C



D



E

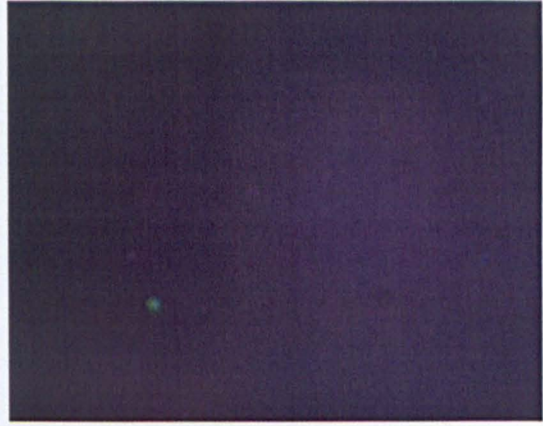
Figure 5-53 Adhesion of human dermal fibroblasts (F002, passage 6) in serum free media to tissue culture plastic after A) 30 minutes, B) 1 hour, C) 2 hours, D) 4 hours and E) 24 hours. Blue represents cell nuclei (DAPI) and green represents F-actin (FITC-phallodin).

Figure 5-54 to Figure 5-59 on the following pages (p103-103) show the cell attachment to poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) hydrogels. In these images the numbers of cells per unit area may not be representative of the entire surface of the polymer but the morphology of the cells is representative of the cells that could be found.

As expected from phase contrast microscopy and MTT assay results, cells did not adhere well to the hydrogel without peptide both in terms of cell number and F-actin organisation. There was little evidence of F-actin surrounding the cells at any of the time points except as a rounded area directly surrounding the nucleus showing the cells were not spread. After 24 hours culture the cells remaining on the polymer had formed into small clusters as shown in Figure 5-54, p103 image D.

On hydrogels containing 0.1 μmol/g of the peptide there were no cells that could be found at the 30 minute time point. However at later time points greater numbers of cells could found on the polymer surface than on the negative control indicating that this low level of peptide concentration was able to promote cell adhesion but that that integrin binding took greater than 30 minutes to become effective. These images are shown in Figure 5-55, p103. There was slight evidence of an F-actin network surrounding the cells at the later time points of 4 and 24 hours but this was much less extensive than for TCP. The F-actin surrounding the cells on these hydrogels were in a "spiky" conformation with tendrils extending out from the cell nuclei rather than forming an orthogonal shape surrounding the nucleus as featured on TCP. The cells on these surfaces were usually found in small clusters, which was also observed by phase contrast microscopy in section 5.6.2.1 (p103, GMMA Polymers containing Long Peptide Sequence). Image C shows a defect in the structure of the polymer, probably caused by rapid swelling or shrinkage when changing the solvent from synthesis conditions to cell culture conditions.

A



B

C

D

Figure 5-54 Adhesion of human dermal fibroblasts (F002, passage 6) in serum free media to poly(GMMA-co-EGDMA) hydrogels after A) 30 minutes, B) 1 hour, C) 4 hours and D) 24 hours. Blue represents cell nuclei (DAPI) and green represents F-actin (FITC-phallodin). Note: no cells were found at the 2 hour time point and so no picture is represented here.

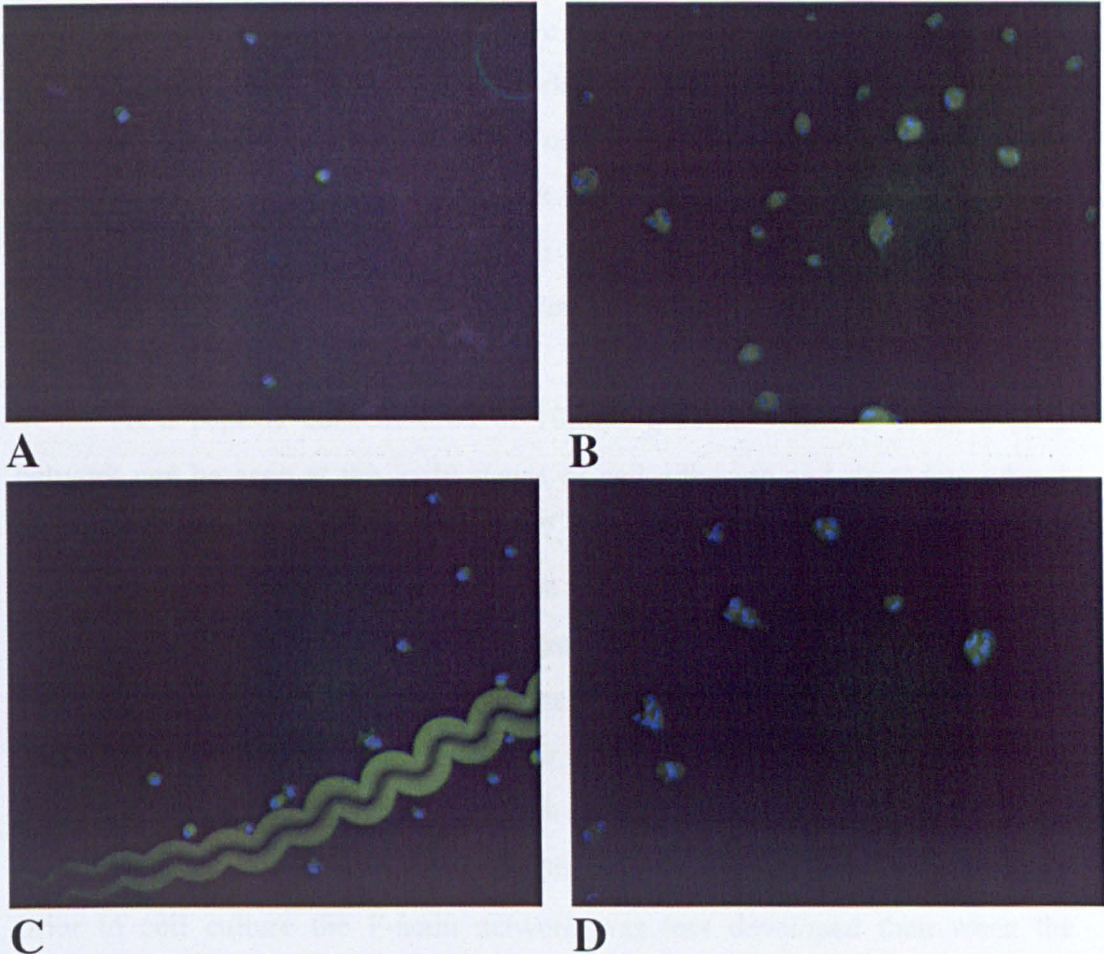
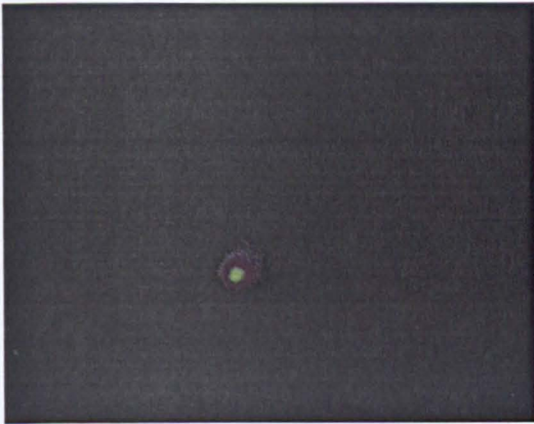


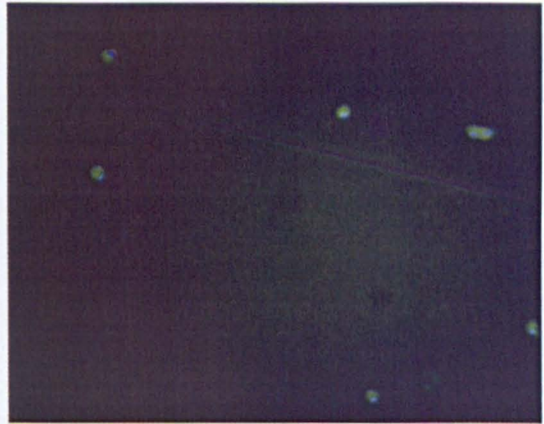
Figure 5-55 Adhesion of human dermal fibroblasts (F002, passage 6) in serum free media to poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) hydrogels containing 0.1μmol/g of peptide and having been pre-treated with 3 days deprotection with GST/GSH. Cells were fixed after A) 1 hour, B) 2 hours, C) 4 hours and D) 24 hours. Blue represents cell nuclei (DAPI) and green represents F-actin (FITC-phallodin). Note: no cells were found at the 30 minute time point and so no picture is represented here.

At a peptide concentration of 1μmol/g there are still very few cells that can be found after 30 minutes of culture as shown in Figure 5-56, p103. Cells show little evidence of an F-actin network up to 4 hours after seeding but at this time point some cells shows extensions of the F-actin network from the nucleus in the "spiky" conformation observed on the hydrogels containing 0.1μmol/g of peptide. After 24 hours of culture the F-actin network has formed a more traditional network surrounding the nucleus but this is much less extensive than that found with cells on TCP.

At a peptide concentration of 10μmol/g some evidence of an F-actin network can be seen at the early stages of cell adhesion and spreading after 1 hour and 2 hours as shown in Figure 5-57, p103. After 4 hours this has developed into the same "spiky" conformation as observed with lower peptide concentrations. After 24 hours cells appear to have clustered together and created F-actin bridges between neighbouring cells. In these cells the F-actin network appears to have formed into a thin linear or broad linear shape. Once again the surface area covered by F-actin is much reduced compared to TCP. When the hydrogels with 10μmol/g of MacGR(4-Bbs)GDS were not deprotected with GST prior to cell culture the F-actin network was less developed than when the peptide was deprotected with GST as shown in Figure 5-58, p103. At the early stages after cell seeding there was little evidence of any F-actin network formation. At 4 and 24 hours after cell seeding there is some evidence of F-actin formation but this was much less extensive than when the peptide had been deprotected. The F-actin again showed a "spiky" conformation especially 24 hours after cell seeding.

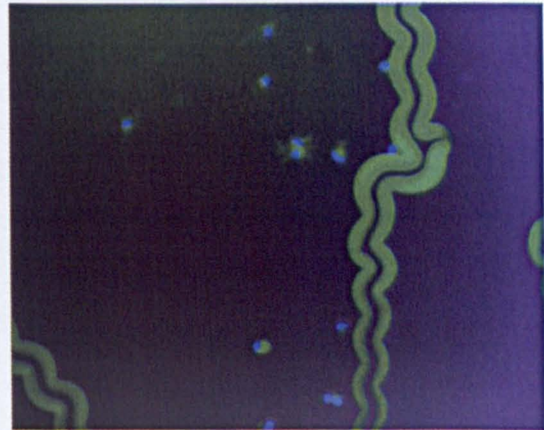


A



B

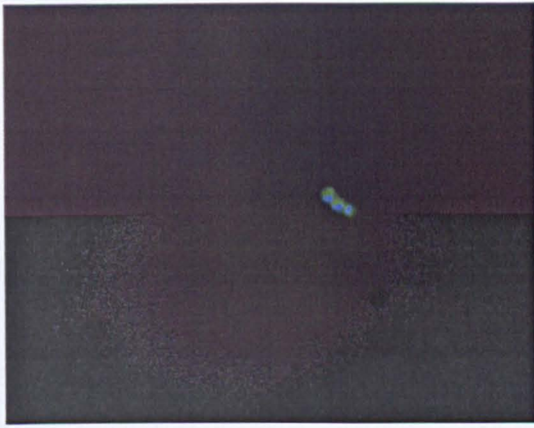
C



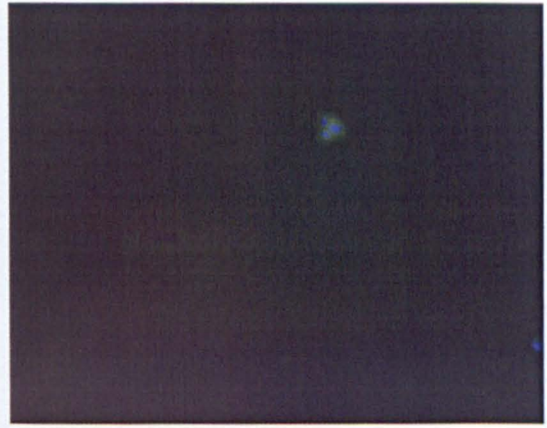
D

E

Figure 5-56 Adhesion of human dermal fibroblasts (F002, passage 6) in serum free media to poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) hydrogels containing 1μmol/g of peptide and having been pre-treated with 3 days deprotection with GST/GSH. Cells were fixed after A) 30 minutes, B) 1 hour, C) 2 hours, D) 4 hours and E) 24 hours. Blue represents cell nuclei (DAPI) and green represents F-actin (FITC-phallodin).

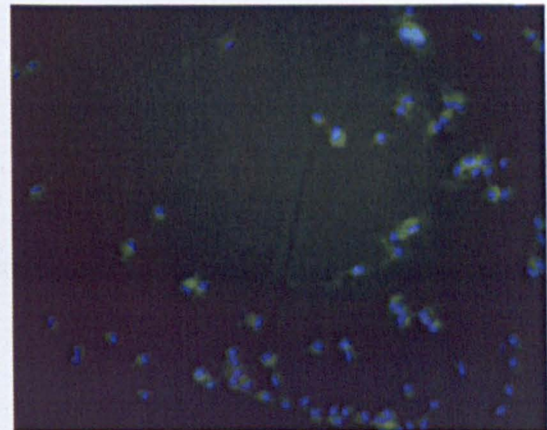


A

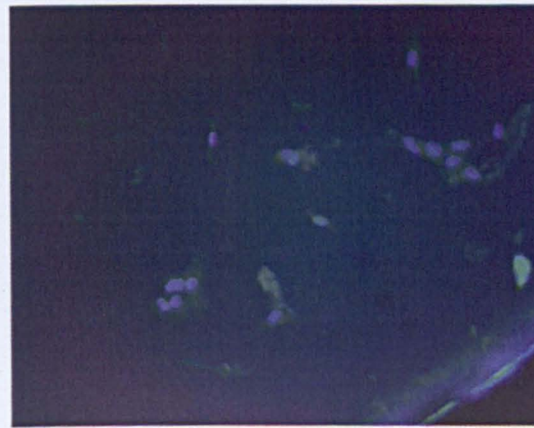


B

C



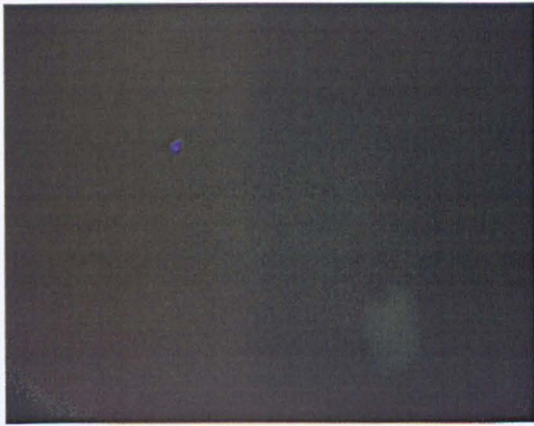
D



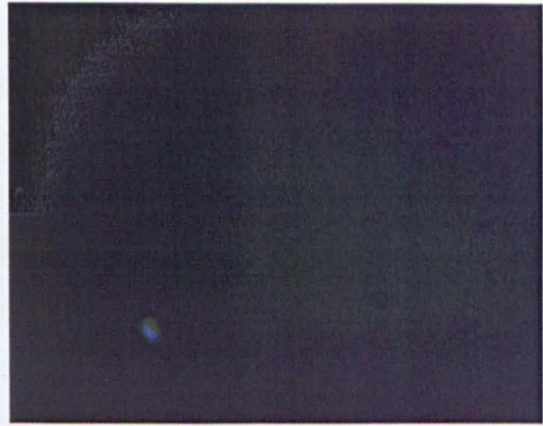
E

F

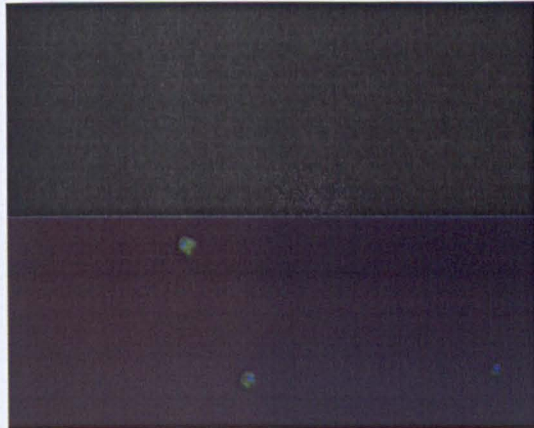
Figure 5-57 Adhesion of human dermal fibroblasts (F002, passage 6) in serum free media to poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) hydrogels containing 10μmol/g of peptide and having been pre-treated with 3 days deprotection with GST/GSH. Cells were fixed after A) 30 minutes, B) 1 hour, C) 2 hours, D) 4 hours and E & F) 24 hours. Blue represents cell nuclei (DAPI) and green represents F-actin (FITC-phallodin).



A

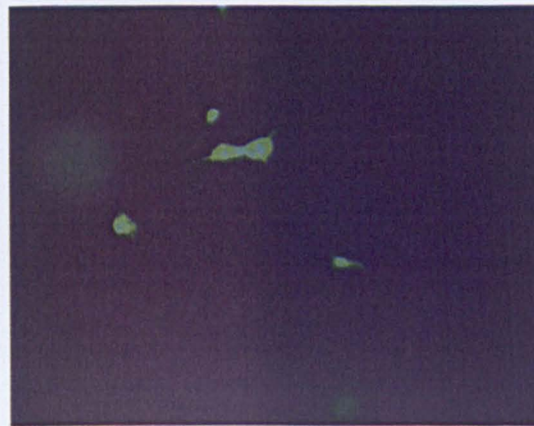


B



B

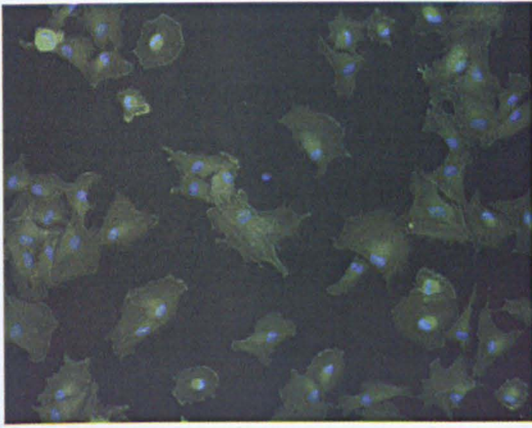
C

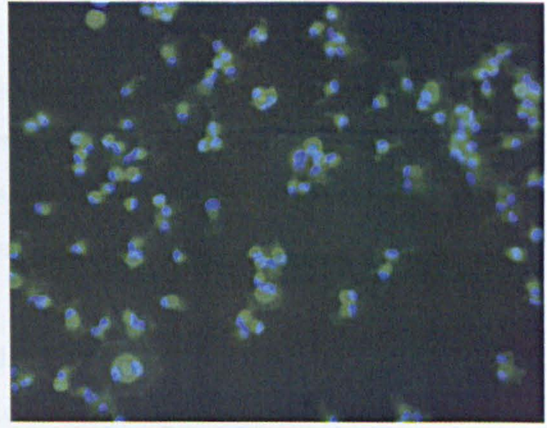


D

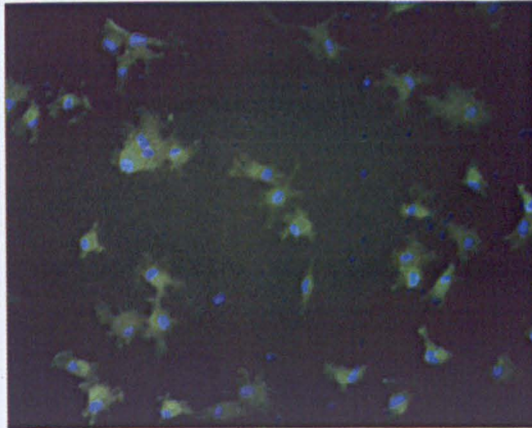
Figure 5-58 Adhesion of human dermal fibroblasts (F002, passage 6) in serum free media to poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) hydrogels containing 10µmol/g of peptide and not pre-treated. Cells were fixed after A) 30 minutes, B) 1 hour, C) 2 hours, D) 4 hours and E) 24 hours. Blue represents cell nuclei (DAPI) and green represents F-actin (FITC-phallodin).

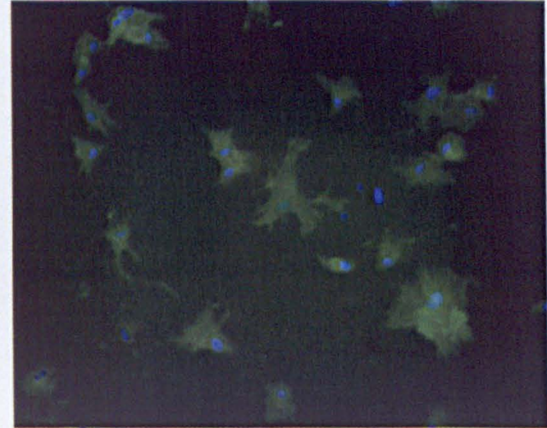
The F-actin organisation of cells of passage number 12 was also investigated on hydrogels containing 10μmol/g of peptide that had been deprotected with GST for 0, 1 or 3 days. The cells were fixed and stained after 4 hours of culture. Cells of passage number 12 on TCP looked identical to cells of passage number 6 on TCP but the cells of passage number 12 on RGD-containing hydrogels had a much greater surface area covered by F-actin compared to the respective polymers cultured with cells of passage number 6 as shown in Figure 5-59, p103. The morphology of the F-actin network was again "spiky" as shown with cells of passage number 6 but the surface area covered and the number of extensions was much greater. The surface area of the polymer covered by the F-actin network appears to increase with increasing amount of deprotection. This would imply that, whilst the cell culture as assessed by MTT assay was not improved by increasing peptide deprotection time, the cell area and / or the F-actin network organisation could be improved by increasing peptide concentration through increasing peptide deprotection time.





A B





C D

Figure 5-59 Adhesion of human dermal fibroblasts (F1833, passage 9) in serum free media to A) TCP or poly(GMMA-co-EGDMA-co-MacGR(4-Bbs)GDS) hydrogels containing 10μmol/g of peptide and pre-treated with B) 0 GST, C) 1 day GST/GSH or D) 3 days GST/GSH. Cells were fixed after 4 hours culture and the nuclei were stained blue with DAPI and F-actin was stained green with FITC-phallodin.

The "spiky" conformation of the cells on polymers containing RGD is probably due to strong integrin binding being made at point contacts. The remainder of the hydrogel is resistant to protein adsorption and cell contact. This leads to the formation of actin filaments spanning from the cell nucleus to areas of RGD-integrin point contact and not spread in the entire area surrounding the cell nucleus. This would also explain why greater numbers of extensions are seen when the RGD concentration is increased. Similar "spiky" F-actin organisation patterns have been observed in other systems utilising RGD where the substrate is highly hydrophilic such as PEG, HEMA[81], hydroxyapatite[210] and PEG/PEO[114].

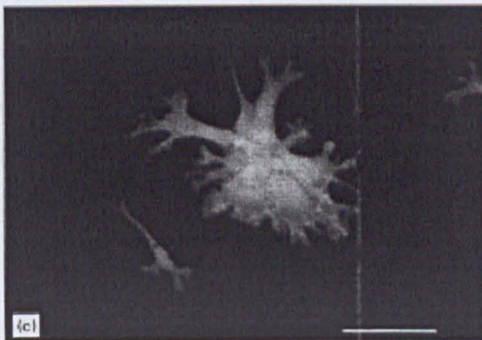


Figure 5-60 Rhodamine phalloidin staining for F-actin microfilament bundles in NIH 3T3 fibbroblasts seeded on PS grafted with GRGDSY using varying amounts of EGAP combined with F108. (c) 1% EGAP ($2.6x10^2$ peptides/cm^2). The size bar corresponds to 62 μm. Reproduced from Neff et al[†,114]

† Reproduction of Figure 6c from "Neff, J. A., Tresco, P. A. & Caldwell, K. D. Surface Modification for the Controlled Studies of Cell-Ligand Interactions. *Biomaterials* **20**, 2377-2393 (1999)" with the permission of Elsevier Ltd.

6 Conclusions & Future Work

The aims of this research were to investigate the effects of inclusion of RGD peptides on the ability of biomaterials to support cell attachment, viability and normal functions for the purposes of tissue engineering. In particular the goals were to determine whether the use of RGD-peptides in a non-integrin selective manner was of value in comparison to and in conjunction with the strategy of promoting protein adsorption. The hypotheses were that the peptide would be effective at increasing the cell attachment and viability on substrates with low native abilities but be ineffective at increasing the attachment and viability of cells on substrates with a high native ability. Hydrogels synthesised from glycerol methacrylate, butyl methacrylate and ethylene glycol dimethacrylate were shown to have varying native abilities to attach and sustain cells and be suitable targets for RGD incorporation studies. The most appropriate method of synthesising the hydrogels was found to be as a thin film formed on the surface of PET by UV initiation due to considerations of handling and scale of reactions.

It was speculated that the extent of the improvement and the point at which the intrinsic level of the ability of a substrate to adhere cells cannot be improved would be affected by the concentration of the peptide. This set the requirement that for effective comparison of substances the peptide concentration should be identical in substrates of different compositions. A review of current literature showed that controlling the peptide concentration by a modification synthesis method would be difficult due to alterations in coupling efficiencies under varying conditions including the nature of the substrate being modified. To keep the concentration of the peptide constant in all materials a copolymerisation method of incorporating RGD was selected. Protection for the side chains of RGD sequences during polymerisation appears to be a requirement for controlling the effective concentration of active moieties as lack of protection reduces the effectiveness of the peptide.

The copolymerisation of an RGD sequence containing standard peptide protecting chemistry with methacrylates was investigated. Linear polymers of glycerol methacrylate acetonide ester were shown to be deprotected by the same conditions required to deprotect the peptide (95% TFA, 4hours) with little side chain hydrolysis. Hydrogels synthesised with ethylene glycol dimethacrylate were shown to be unstable towards TFA and the cross-linker was replaced with divinylbenzene. Hydrogels were synthesised containing the protected peptide but the TFA treatment was found to fracture the hydrogels to such an extent that they were unsuitable for cell culture purposes.

The use of enzymatically cleavable protecting groups for the side chains of arginine was investigated. Arginine derivatised with the 4-bromobenzyl sulphonyl (4-Bbs) protecting group on the side chain was synthesised and purified. The removal of 4-Bbs by Glutathione-S-Transferase (GST) was investigated and it appeared that the reaction was first order with respect to both the GST concentration and the concentration of the amino acid. It was also found that the reaction reached equilibrium after 24 hours at 37°C. The equilibrium position and hence the extent of deprotection depended on the protecting group utilised for the amine terminus of the arginine. It was found that 87% deprotection was achieved when Z was used but only 23% deprotection was achieved when Fmoc was used as the N-terminus protecting group. It was hoped that, when the peptide was immobilised in a solid, the deprotection reaction would be driven to completion by the heterogeneous nature of the reaction.

Peptides containing the enzymatically cleavable protected arginine were synthesised and utilised to make a library of polymers with glycerol methacrylate and butyl methacrylate. In serum-containing media glycerol methacrylate based polymers containing RGD either with or without a spacer arm were shown to promote increased cell spreading at higher concentrations of RGD (1-10μmol/g) but the viability assays showed no significant increase in cell number and activity. Under the same conditions no differences between peptide containing butyl methacrylate hydrogels and non-peptide containing controls could be discerned by both visual methods and viability assay based methods. To determine whether the peptide had been fully deprotected after 24 hours reaction with GST the behaviour of cells on RGD-containing materials after 0, 1 and 3 days deprotection with GST was examined. There were no significant differences

between the cell behaviours on materials that had been deprotected for 1 and 3 days. However there were also no significant differences between the polymers containing peptide that had not been deprotected and those that had. It was found that serum-containing media possessed the ability to deprotect the arginine side chain of the peptide, probably due to GST or other enzymes in the fetal calf serum.

In serum-free culture concentrations of 1 or 10μmol/g of peptide (with and without a spacer arm) in glycerol methacrylate hydrogels after 1 days GST treatment promoted significant improvement in cell viability and cell spreading. The presence of a spacer arm is not necessary for RGD to be effective in random cross-linked polymers that are synthesised from small monomeric units. It would be interesting to see whether the spacer arm was necessary in block polymers synthesised from oligomeric glycerol methacrylate units such as utilised in the research by Sun et al[192]. There was no significant alteration in both the cell spreading and the cell viability on butyl methacrylate hydrogels when any of the peptides were incorporated at any of the concentrations tested. It seems likely that the native ability of the BMA gels to promote cell adhesion and viability puts these materials above the non-improvement point where RGD is effective with the peptide-integrin affinity tested and the concentrations utilised. However there are some concerns over the continued presence of the 60μm coating during cell culture as the coating could have become detached during washing procedures.

The analysis of cell culture on glycerol methacrylate materials that had been subjected to different length of time of GST deprotection showed that for large concentrations (10μmol/g) maximal cell adhesion occurred after 1 days deprotection and further deprotection (3-5 days) did not increase the effect of the RGD peptide. For an intermediate concentration (1μmol/g) of peptide in glycerol methacrylate hydrogels the number and viability of cells cultured on the polymers were linearly dependent on the number of days deprotection. Low concentrations of peptide (<0.1μmol/g) did not promote an increase in cell number or viability even after 5 days deprotection with GST. At the 10μmol/g concentration of peptide the cell number and viability was increased over the non-peptide containing control, therefore either the peptide has a non-integrin

specific effect on the ability of the polymer to adhere cells or the peptide has been deprotected by the culture conditions.

To investigate whether peptides were deprotected by culture conditions glycerol methacrylate hydrogels containing the protected peptide sequence without a spacer arm were incubated with non-contacting cells in co-culture baskets. The adhesion and viability of cells on hydrogels containing 1µmol/g was significantly increased by pre-treatment with non-contacting cells. A significant improvement in cell adhesion and viability was also observed for TCP after pre-treatment with non-contacting cells, which is likely to be due to the adsorption of cell derived proteins. No improvement was observed for non-peptide containing controls, which is assumed to be due to lack of ability of the glycerol methacrylate hydrogels to adsorb cell derived proteins. The adhesion and viability of cells on hydrogels containing 10µmol/g was not significantly increased by pre-treatment with non-contacting cells however under the same conditions no improvement was observed from pre-treatment with GST either. This is assumed to be due to a maximum cell response derived from peptide deprotection when cells were seeded on non-treated and treated polymers alike. From the non-improvement after pre-treatment with non-contacting cells on the polymers with high peptide concentration it can be concluded that no protein adsorption occurs on any glycerol methacrylate hydrogels regardless of the peptide concentration. Thus the improvement in the hydrogels containing 1µmol/g of peptide after pre-treatment with non-contacting cells is due to deprotection of the peptide by soluble cell derived products e.g. GST.

The promotion of cell adhesion and viability to GMMA hydrogels containing RGD peptides was more clearly observed at higher passage numbers as the relative cell adhesion and viability was reduced on TCP but maintained on the peptide-containing hydrogels. Consequently the results of cell culture on GMMA hydrogels containing 10µmol/g of RGD gave 120% for passage 12 but only 60% for passage 6 when expressed as a % of the TCP control via the MTT assay.

The staining of cells on glycerol methacrylate hydrogels containing MacGR(4-Bbs)GDS showed the shape of the F-actin network on the peptide-containing hydrogels was "spiky" compared to the orthogonal shape shown on TCP. It also showed that that the F-actin network was much less developed and covered a smaller area on the hydrogels compared to TCP. Cells of passage number 12 produced an F-actin network with a larger surface area than cells of passage number 6.

It was shown that the incorporation of the vinyl containing peptide into GMMA hydrogels was low and that the immobilised amounts of peptide were in the order of 13-32% that of the nominal peptide concentration. The percentage of input peptide that was immobilised was dependent on the input concentration and decreased with increasing peptide concentration. Swelling of the hydrogel in aqueous media meant that the concentration of the peptide was further diluted over a larger volume to give effective peptide concentration of 6-13% that of the nominal peptide concentration. The deprotection of the arginine side chain was not completely achieved by treatment with GST and consequently the active peptide concentration is lower than the immobilised peptide concentration. The actual amount of deprotected, active peptide could not be determined as the peptide was deprotected by cells, both by cells seeded on the surface and by non-contacting cells in co-culture baskets. Significant improvements in cell adhesion, spreading and viability were observed in GMMA polymers with nominal concentrations over 1μmol/g, which is equal to or less than 0.013μmol/g active concentration. Maximal cell adhesion, spreading and viability was observed on GMMA polymers with nominal peptide concentrations of 10μmol/g, which is equal to or less than 0.13μmol/g active concentration. Assuming an integrin penetration depth of 10nm these results would correspond to significant improvements in cell adhesion, spreading and viability at less than 0.013 pmol/cm^2 and maximal cell adhesion, spreading and viability at less than 0.13 pmol/cm^2.

In contrast no improvement in cell adhesion, viability or spreading was observed on BMA hydrogels containing any of the peptide concentrations tested (up 10μmol/g nominal concentration). This may be due to decreased active peptide concentration compared to the GMMA polymers via reduced incorporation efficiency or reduced deprotection efficiency. Alternatively the

lack of improvement in BMA hydrogels may be due to the need for larger concentrations of peptide to promote cell adhesion or because protein adsorption masks the peptide and prevents integrin binding. The active peptide concentration as perceived by cells cannot be determined and so the reason for the non-improvement in cell adhesion and viability on incorporation of this peptide in BMA hydrogels also cannot be determined by this study. This unfortunately means that several of the questions posed in the hypotheses are unable to be answered. This is a limitation of this method of producing RGD-modified polymers. However the fact that the peptide utilised promotes maximal levels of cell adhesion and viability at concentrations lower than those reported in many other studies shows that the protection strategy has preserved the activity of the peptide that has been lost in other polymerisation methods. In addition the bulk derivatisation means that inaccuracies in determining the depth of modification have been eliminated, which is probably why the peptide concentration required for maximal cell adhesion is lower than that reported for substrates synthesised by modification procedures to polymers. Therefore this strategy has some advantages but it could be improved by finding alternative enzymatically cleavable protecting groups that can be removed with 100% efficiency. Benzyl sulphonamides with electron withdrawing groups ortho/para to the sulphonyl are known to be removed by GST and using a substituent that is more electron withdrawing that benzene and using more substituents should make the removal more effective. A likely candidate for high deprotection efficiency would be the 2,4,6-trinitrobenzyl sulphonyl group. As well as the removal of arginine side chain residues with GST the protection of other side chains followed by enzymatic removal should be possible. Protection of carboxylic acid side chains such as those that are found in glutamic acid and aspartic acid could be achieved with by esterification. Enzyme catalysed hydrolysis of esters is a well documented technique and it should be possible to find an enzyme / ester combination that could be utilised as a protection strategy for RGD peptides. Ideally the removal of all protecting groups could be achieved in one step with a enzyme cleavage cocktail.

Whilst the strategy of using enzyme deprotection to achieve copolymerisable RGD peptides has been successful and produced materials that may be suitable for tissue engineering where the peptide has improved cell adhesion, spreading and viability it has not been possible to study the effect of peptide concentration in different substrates. The effect of varying peptide concentration when immobilised against backgrounds of different abilities to adsorb proteins would be a valuable contribution to background knowledge for tissue engineering. The easiest method to perform this investigation would be to modify polymers containing carboxylic group with RGD peptides via N-hydroxysuccinimide activation. The immobilisation of the peptide into different substrates would probably give different concentrations for the same input concentration. However utilisation of a large range of input concentrations followed by determination of the peptide concentration should result in polymers of different compositions but similar peptide concentrations being available for comparison. In addition the investigation should look at the cell adhesion, viability and other functions at various time points and utilise different primary cell types, which are weaknesses of the current investigation.

7 Materials and Methods

7.1 Monomer Synthesis

7.1.1 Synthesis of GMAc

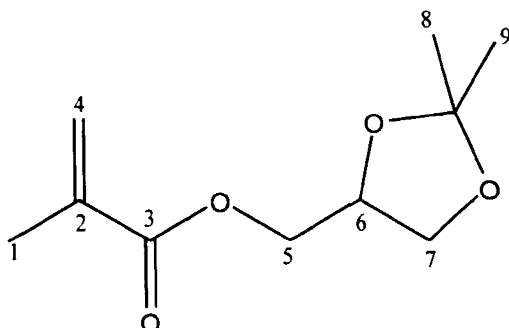


Figure 7-1 2-methyl-acrylic acid 2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester (GMAc)

Solketal was purchased from Sigma-Aldrich and was mixed with toluene and then rotary evaporated at 70°C for 1 hour to remove toluene, water and other impurities before use. All other reagents were purchased from Sigma-Aldrich and used without further purification. Dry solketal (1eq) and (1.6eq) of triethylamine (TEA) was mixed with DCM, placed under nitrogen and cooled to 0°C. Methacryloyl chloride (1.2eq) was added drop wise and reaction was left overnight before being quenched with H_2O. The organic phase was separated and DCM removed by rotary evaporation. The crude GMAc was purified by distillation under high vacuum (b.p. = 44°C) and stored and -8°C until use. A typical reaction used 330g (2.5 mol) solketal, 400g (4.0 mol) triethylamine and 310g (3.0 mol) methacryloyl chloride with 1L DCM and 1L H_2O. Purity of the product was determined by ^{1}H NMR.

^{1}H NMR: δ 1.25 (3H, s, -C^8**H_3**), 1.35 (3H, s, -C^9**H_3**), 1.85 (3H, s, -C^1**H_3**), 3.70 (1H, m, -C^5(**H**)H-), 3.95 (1H, m, -C^5(H)**H**-), 4.10 (2H, d, J=5.5Hz, -C^7**H_2**-), 4.25 (1H, m, -C^6**H**-), 5.50 (1H, s, =C^4**H_A**H_B), 6.05 (1H, s, =C^4H_A**H_B**)

7.2 Polymer Synthesis

7.2.1 Materials

Glycerol methacrylate was purchased from Melrob Service Ltd, UK and used without further purification. Butyl methacrylate was purchased from Sigma-

Aldrich and purified by distillation under reduced pressure prior to use. Azobisisobutironitrile (AIBN) was purchased from Sigma-Aldrich and re-crystallised from methanol prior to use. All other reagents were purchased from Sigma-Aldrich and used without further purification.

7.2.2 Linear Polymer Synthesis

Linear polymers of 2-methyl-acrylic acid 2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester and butyl methacrylate were synthesised in an ampoule using the freeze-pump-thaw method. The monomer or monomers were mixed with IPA or methanol and 1 wt % of AIBN. The solution was then placed in an ampoule and frozen with liquid nitrogen. The ampoule was evacuated under high vacuum and then the solution was allowed to thaw. This was freeze-pump-thaw process was repeated 3 times and then the ampoule sealed whilst under vacuum. The ampoule was then placed in a water bath at 60°C for 20-24 hours. The contents of the ampoule precipitated into diethyl ether, re-dissolved in ethanol and then re-precipitated into diethyl ether. A typical reaction involved 3.0106g of GMAc, 0.3105g of AIBN and 50ml of IPA.

7.2.3 Hydrogel Synthesis by Thermal Initiation

Solutions of monomers (glycerol methacrylate, butyl methacrylate), cross-linker (ethylene glycol dimethacrylate) and 1 wt % AIBN were placed in a sealed container with propan-2-ol (1:1 mass: volume) and purged with nitrogen for a minimum of 20 minutes. The solutions were syringed into moulds and then placed in an oven at 60°C overnight. Stock solutions were made up as per Table 5-1 and were used for all hydrogels made by thermal initiation.

Polymer	GMA Mass / g	BMA Mass / g	EGDMA Mass / g	AIBN Mass / g	IPA Volume / ml
100% GMA	22.5694	0	1.1356	0.2250	22.5
75 % GMA, 25% BMA	14.2864	4.7673	0.9668	0.1910	19
50% GMA, 50% BMA	13.9376	13.9948	1.3974	0.2789	28
25% GMA, 75% BMA	7.3848	22.1425	1.4743	0.3001	30
100% BMA	0	20.8981	0.5229	0.2099	20

Table 7-1 Stock solutions used to make hydrogels synthesised by thermal intitiation.

7.2.4 Hydrogel Synthesis by UV Initation

All UV curing was conducted in a Dymax bond box with a medium pressure mercury lamp. The intensity of this system has been determined to be 30mM/cm^2 at 400nm at a distance of 3mm below the reflector.

Method 1:

Stock solutions of monomers and cross-linkers (as shown in Table 7-2) were mixed with a solution of 1wt % initiator (2-hydroxy-2-methylpropiophenone) in IPA were purged with nitrogen for a minimum of 20 minutes. The solution was syringed into a mould, placed in a dymax bond box and irradiated with ultraviolet light for 40 seconds on each side and turned 3 times (total irradiation time 160 seconds)

Method 2:

Solutions of monomers (GMMA, BMA, LMA, HEMA, DMAEM or styrene), cross-linker (EGDMA or DVB) and 1 wt % initiator (2-methyl-4'-(methylthio)-2-morpholinopropiophenone (MMP), 2,2-dimethoxy-2-phenylacetophenone (DPP), 2-chlorothioxanthen-9-one (CTX) 2-hydroxy-2-methylpropiophenone (HMPP) or a 50:50 blend of diphenyl(2,4,6-trimethylbenzoyl) phosphine oxide (DPO) and HMPP) in a 50:50 mixture of glycerol and ethanol were placed in a multiwell plate. The multi-well plate was placed inside a box with a quartz window. The box was placed under vacuum and then nitrogen repeatedly. After a minimum of 3 cycles the box was placed in a Dymax bond box where it was irradiated with ultraviolet light for a maximum of 5 minutes (200 seconds)

Method 3:

Solutions of monomers (GMMA, BMA or combinations thereof), 2.5 weight % cross-linker (EGDMA) and 1 wt % initiator 2-methyl-4'-(methylthio)-2-morpholinopropiophenone (MMP), 2,2-dimethoxy-2-phenylacetophenone (DPP), 2-chlorothioxanthen-9-one (CTX) 2-hydroxy-2-methylpropiophenone (HMPP) or a 50:50 blend of diphenyl(2,4,6-trimethylbenzoyl) phosphine oxide (DPO) and HMPP) were dissolved in methanol and purged with nitrogen for 20 minutes in a covered vessel. Typically large scale stock solutions of monomers and cross-linkers (as shown in Table 7-2) were made up and stored at -8°C until use when they were warmed up to room temperature and weighed before a solution of 1% vol/vol or weight/vol initiator was added at a ratio of 1ml per g of

monomer solution. The solutions were syringed onto a sheet of material and drawn down the sheet using a 60 micron K-Bar. The sheet was then placed a box with a quartz window. The box was placed under vacuum and then nitrogen repeatedly. After a minimum of 3 cycles the box was placed in a Dymax bond box where it was irradiated with ultraviolet light for a maximum of 5 minutes (200 seconds).

Polymer	GMA Mass / g	BMA Mass / g	EGDMA Mass / g
100% GMA	49.2333	0	1.2483
75 % GMA, 25% BMA	46.9695	15.6486	1.5654
50% GMA, 50% BMA	40.3614	40.317	2.0472
25% GMA, 75% BMA	25.4892	76.6554	2.5353
100% BMA	0	45.0109	1.1249

Table 7-2 Stock solutions of monomers used for UV initiated polymerisations.

7.3 Polymer Reactions

7.3.1 Reaction of Linear Polymers with TFA

The dried linear polymers of GMAc and BMA produced in section 7.2.2 were dissolved in neat TFA (20 ml/g) and stirred for 1 or 4 hours at room temperature and pressure. A typical reaction used 1.2041g of poly(GMAc) and 24ml of TFA. The reaction mixtures were precipitated in diethyl ether, filtered, re-dissolved in methanol and then re-precipitated into diethyl ether again before being dried and analysed by NMR.

7.3.2 Reaction of Cross-linked Polymers with TFA

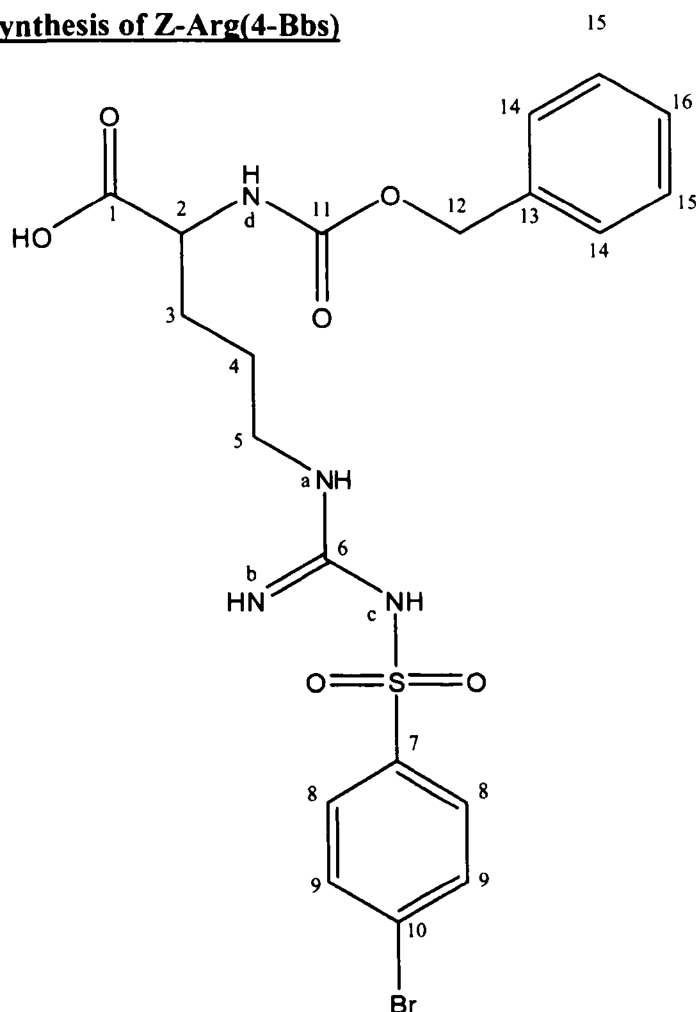
Disks with a diameter of 1.5 cm were cut from sheets of thermally cured polymers synthesised by the protocols mentioned in section 7.2.3. The disks were washed three times with ethanol and half the disks were dried in a vacuum dessicator. Swollen disks had excess ethanol blotted from the surface before being placed in TFA (20 ml/g approx.) whereas dried disks were placed directly into TFA (20 ml/g exact). The disks were allowed to react with the TFA for 4 hours before they were removed with tweezers and rinsed with ethanol. All disks were then washed three times with ethanol.

7.4 Amino Acid Synthesis

7.4.1 Materials

All reagents were purchased from Sigma-Aldrich and used without further purification.

7.4.2 Synthesis of Z-Arg(4-Bbs)



N-Carbobenzoxy-L-Arginine (Z-Arg, 1eq) was dissolved in 3M NaOH(aq) (5ml / g) and acetone (15ml / g) and then cooled to 0°C. 4-bromobenzylsulphonylchloride (3-4eq.) was dissolved in acetone (5ml / g) and added drop wise. A typical reaction used 5.0388g Z-Arg-OH, 12.2241g 4-bromobenzylsulphonyl chloride, 15ml of 3 mol dm^{-3} $NaOH_{(aq)}$ and a total of 125ml acetone. The reaction continued for 2 hrs at 0°C followed by 2 hrs at RT during which time the pH was monitored and kept at pH 9 or above. The final reaction mixture was acidified to ~pH 6.5 with saturated citric acid solution and the acetone removed by rotary evaporation. Deionised water (10ml /g) was added and the solution further acidified to ~pH3 before the product was extracted into

ethyl actetate. The organic fractions were combined, washed with water and brine and then reduced in volume to approx. 3ml /g. The product was precipitated into ice cold diethyl ether and then left to solidify at -8°C before the solvent was decanted off and the final traces of solvent removed under high vacuum. 40-50% yield).

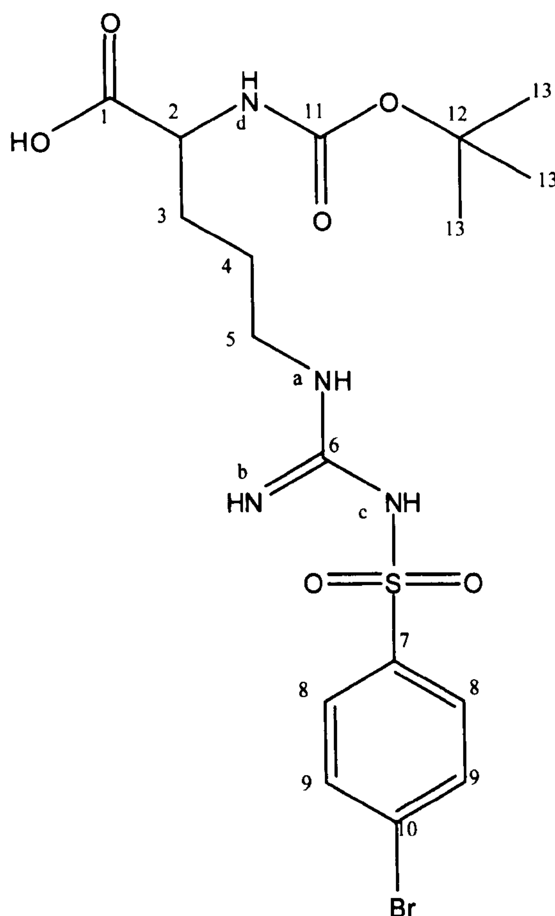
^{1}H NMR: δ 1.55 (2H, m, -C^{4}**H**$_2$-), 1.80 (2H, m, -C^{3}**H**$_2$-), 2.15 (2H, m, -C^{5}**H**$_2$-), 3.20 (2H, broad s, -N^{b}**H**- / -N^{a}**H**-) 3.35 (1H, broad s, -N^{d}**H**-), 3.75 (1H, broad s, -N^{c}**H**-), 4.15 (1H, m, -C^{2}**H**-), 5.10 (2H, m, -C^{12}**H**$_2$-), 7.20-7.40 (5H, m, aromatic H's), 7.65-7.80 (4H, m, aromatic H's).

^{13}C NMR: δ 27.1 (C^{4}), 29.9 (C^{3}), 36.9 (C^{5}), 40.7 (C^{2}), 110.6 (C^{12}), 120.7 – 129.9 (aromatic C's, C$^{7-10}$ + C$^{13-16}$), 144.2 (C^{11}), 157.3 (C^{6}), 174.5 (C^{1}).

Elemental Analysis; C: 42.74, H: 4.97, N: 9.36, Br: 15.44, S: 6.46 (expected C: 45.6, H: 4.18, N: 10.6, Br: 15.20, S: 6.08).

HPLC-MS ES^{+}: elution time 16.53 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm), mass peaks; 530, M(^{81}Br^{13}C)H^{+} (25%); 529, M(^{81}Br)H^{+} (100%); 528, M(^{79}Br^{13}C)H^{+} (23%); 527, M(^{79}Br)H^{+} (92%).

7.4.3 Synthesis of Boc-Arg(4-Bbs)



N-Boc -L-Arginine (Boc-Arg, 1eq) was dissolved in 3 mol dm^{-3} $NaOH_{(aq)}$ (5ml/g) and dioxane (10ml/g) and then cooled to 0°C. 4-bromobenzylsulphonylchloride (3-4eq.) was dissolved in 1,4-dioxane (5ml/g) and added drop wise. The reaction continued for 2 hrs at 0°C followed by 2 hrs at RT during which time the pH was monitored and kept at pH 9 or above. The final reaction mixture was acidified to ~pH 6.5 with saturated citric acid solution and the solvent removed by rotary evaporation. Deionised water (10ml /g) was added and the solution further acidified to ~pH3 before the product was extracted into ethyl actetate. The organic fractions were combined and the product was extracted in 5% Na_2CO_3. The aqueous fractions were combined, acidified to pH 3 and then the product was extracted back into ethyl acetate. The ethyl acetate fractions were combined, reduced to ~5% of the original volume and the product was precipitated into diethyl ether. The final white solid was filtered and dried

under vacuum. A typical reaction used 5.0093g of Boc-Arg-OH, 11.0123g of 4-Bbs-Cl, 25 ml of 3 mol dm^{-3} $NaOH_{(aq)}$ and a total of 75ml dioxane.

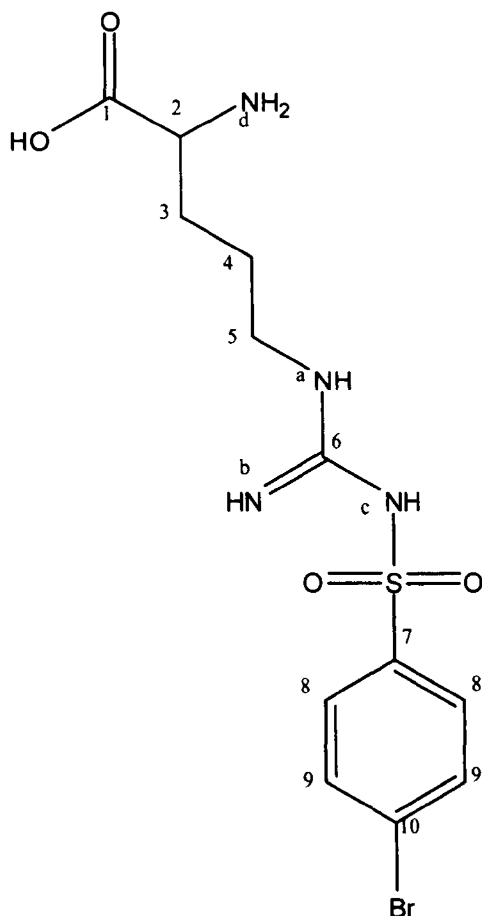
^{1}H NMR: δ 1.45 (9H, s, -C^{13}**H_3**), 1.50-1.90 (4H, m, -C^{3}**H_2**- / -C^{4}**H_2**-), 2.05 (2H, m, -C^{5}**H_2**-), 3.15 (2H, broad s, -N^{b}**H**- / -N^{a}**H**-) 3.35 (1H, broad s, -N^{d}**H**-), 3.75 (1H, broad s, -N^{c}**H**-), 4.10 (1H, m, -C^{2}**H**-), 7.55-7.80 (4H, m, aromatic H's).

^{13}C NMR: δ 26.6 (C^4), 28.7 (C^{13}), 30.1 (C^3), 48.0 (C^5), 57.8 (C^2), 81.2 (C^{12}), 127.8 (C^{10}), 128.9 (C^8), 132.8 (C^7), 133.0 (C^9), 155.2 (C^{11}), 165.0 (C^6), 175.9 (C^1).

Elemental Analysis; C: 38.44, H: 4.81, N: 10.97, Br: 15.73, S: 6.46 (expected C: 41.38, H: 5.11, N: 11.36, Br: 16.20, S: 6.50).

HPLC-MS ES^+: elution time 15.60 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm), mass peaks; 496.3, $M(^{81}Br^{13}C)H^+$ (17%); 495.3, $M(^{81}Br)H^+$ (95%); 494.3 $M(^{79}Br^{13}C)H^+$ (23%); 493.3, $M(^{79}Br)H^+$ (100%).

7.4.4 Synthesis of H-Arg(4-Bbs)



7.4.4.1 Removal of Z from Z-Arg(4-Bbs) with Pd/H_2

Z-Arg(4-Bbs) (1.0566g) was dissolved in methanol (20 ml) and 200mg of palladium supported on carbon was added, the flask was then repeatedly degassed and purged with nitrogen. A hydrogen atmosphere was introduced and the reaction was allowed to stir overnight. The product was precipitated into ether and analysed by HPLC-MS.

7.4.4.2 Removal of Z from Z-Arg(4-Bbs) with HBr/AcOH

Z-Arg(4-Bbs) was dissolved in HBr/AcOH (20ml/g) and allowed to react at room temperature for 2 hours. The volume of liquid was reduced to ~10% by distillation under reduced pressure and the product was precipitated into diethyl ether. H-Arg(4-Bbs) was triturated with 3 x 100ml diethyl ether to remove traces of HBr/AcOH. A typical reaction used 3.0810g of Z-Arg(4-Bbs) and 60ml of HBr/AcOH.

7.4.4.3 Removal of Boc from Boc-Arg(4-Bbs) with TFA

Boc-Arg(4-Bbs) was dissolved in TFA (20ml/g) and allowed to react at room temperature for 2 hours. The volume of liquid was reduced to ~10% by distillation under reduced pressure and the product was precipitated into diethyl ether. H-Arg(4-Bbs) was triturated with 3 x 100ml diethyl ether to remove traces of TFA. A typical reaction used 3.0981g of Boc-Arg(4-Bbs) and 60ml of TFA.

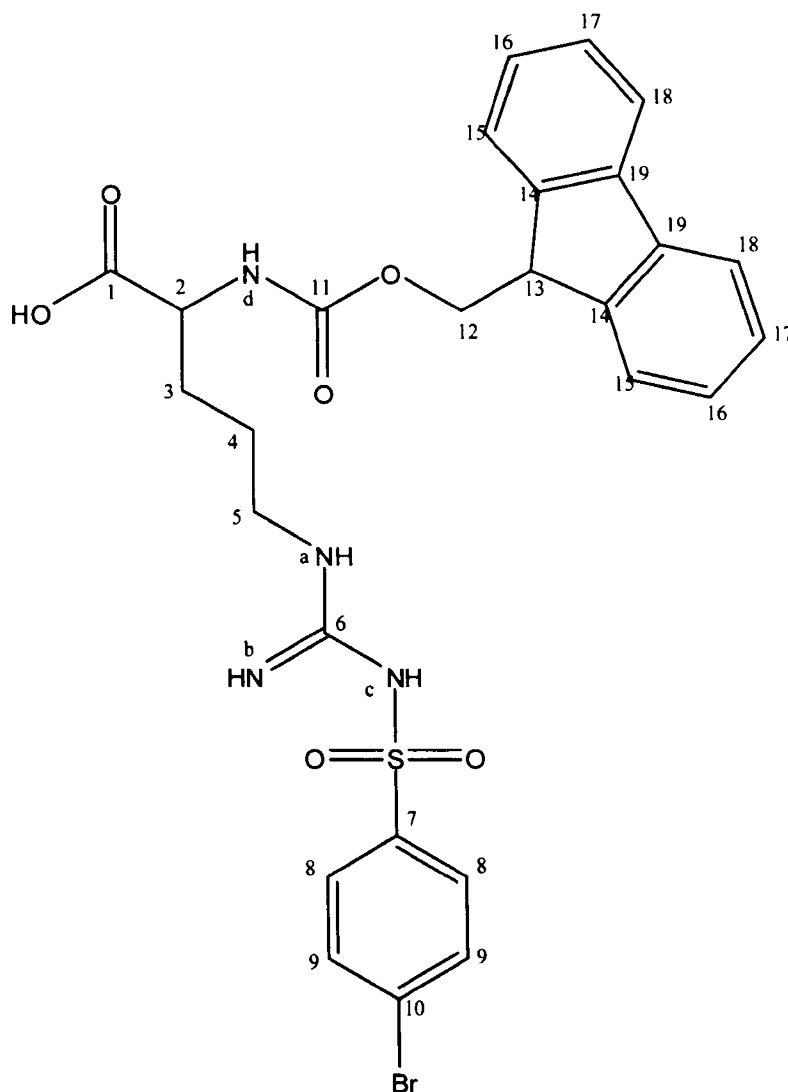
^{1}H NMR: δ 1.15 (2H, t, -C^{3}**H**$_2$-, J=7.5Hz), 1.65 (2H, s, -C^{4}**H**$_2$-), 1.95 (2H, s, -C^{5}**H**$_2$-), 2.95 (2H, broad s, -N^{b}**H**- / -N^{a}**H**-) 3.15 (1H, broad s, -N^{d}**H**-), 3.55 (1H, broad s, -N^{c}**H**-), 4.05 (1H, m, -C^{2}**H**-), 7.85-8.05 (4H, m, aromatic H's).

^{13}C NMR: δ 26.6 (C^{4}), 27.3 (C^{3}), 48.5 (C^{5}), 51.7 (C^{2}), 125.5 (C^{10}), 127.7 (C^{8}), 128.7 (C^{7}), 131.2 (C^{9}), 156.7 (C^{6}), 170.9 (C^{1}).

Elemental Analysis; C: 34.51, H: 4.36, N: 13.11, Br: 20.54, S: 8.01 (expected C: 36.65 , H: 4.36, N: 14.25, Br: 20.32, S: 8.15).

HPLC-MS ES^{+}: elution time 15.60 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm), mass peaks; 496.3, M(^{81}Br^{13}C)H^{+} (17%); 495.3, M(^{81}Br)H^{+} (95%); 494.3 M(^{79}Br^{13}C)H^{+} (23%); 493.3, M(^{79}Br)H^{+} (100%).

7.4.5 Synthesis of Fmoc-Arg(4-Bbs)



H-Arg(4-Bbs)-OH (2.49g, 5.65mmol) was dissolved in 6% aqueous sodium carbonate (21ml) and the solution was cooled to 0°C. A solution of 9-fluorenylmethyl succinimidyl carbonate (fmoc-Cl),(1.92g, 5.66mmol) in dioxane (10ml) was added drop wise and the reaction left to stir for 1h with the ice bath removed. The solution was diluted with water (100ml) and washed with ether (2 X 50ml) before acidification with saturated citric acid solution (30ml). The solution was extracted with ethyl acetate (3 X 100ml) and the combined extracts were washed with water (X 2), before drying over $MgSO_4$. The dried solution was concentrated in vacuo and the desired product precipitated by the addition of diethyl ether (3.3g, 89%).

^{1}H NMR: δ 1.30 (2H, m, -C^4**H_2**-), 1.60 (2H, m, -C^3**H_2**-), 2.15 (2H, m, -C^5**H_2**-), 3.25 (2H, broad s, -N^b**H**- / -N^a**H**-) 3.30 (1H, broad s, -N^d**H**-), 3.70 (1H, broad s, -N^c**H**-), 4.05 (1H, m, -C^2**H**-), 4.20 (1H, m, -C^{13}**H_2**-), 4.35 (2H, d,

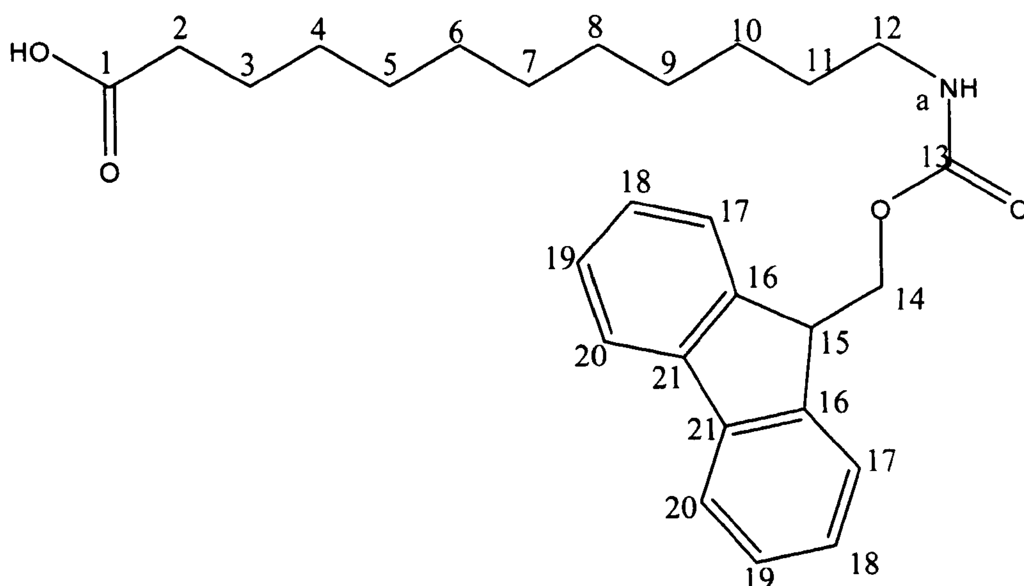
$J_{C12\text{-}C13}$=6.9Hz, -$C^{12}H_2$-), 7.20-7.40 (6H, m, aromatic H's), 7.65-7.80 (4H, m, aromatic H's), 7.95 (2H, d, aromatic H's).

^{13}C NMR: δ 29.9 (C^4), 31.5 (C^3), 45.3(C^5), 55.0 (C^2), 67.7 (C^{12}), 127.1 – 133.0 (aromatic C's, $C^{7\text{-}10}$ + $C^{13\text{-}19}$), 144.2 (C^{11}), 157.3 (C^6), 174.5 (C^1).

Elemental Analysis; C: 51.86, H: 4.39, N: 9.03, Br: 14.81, S: 6.10 (expected C: 52.69, H: 4.42, N: 9.10, Br: 15.73, S: 6.46).

HPLC-MS ES$^+$: elution time 15.60 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). Mass peaks; 619.2, M(^{81}Br^{13}C$_2$)H$^+$ (9%); 618.2, M(^{81}Br^{13}C)H$^+$ (32%); 617.2, M(^{81}Br)H$^+$ (100%); 616.2 M(^{79}Br^{13}C)H$^+$ (29%); 615.2, M(^{79}Br)H$^+$ (86%).

7.4.6 Synthesis of Fmoc-ADDA



12-aminododecanoic acid (ADDA) was suspended in a 10% $Na_2CO_{3\,(aq)}$ solution and cooled to 0°C. 1 eq of Fmoc-Cl in dioxane (10ml per g) was added[273]. The reaction was allowed to warm up to room temperature and stirred for 4 hours. The dioxane was removed by rotary evaporation and water (50ml per g of ADDA) was added. The solution was acidified to pH 3 by addition of concentrated HCL. The product (Fmoc-ADDA) was extracted into ethyl acetate that was then dried with magnesium sulphate and the solvent removed to give the crude product. This was re-crystallised from acetonitrile to form the pure product. A typical reaction used 5.0675g of 12-aminododecanoic acid and 6.1505g of Fmoc-Cl with 125ml of 10% $Na_2CO_{3\,(aq)}$ and 50ml of dioxane.

^{1}H NMR: δ 1.25-1.60 (18H, m, -CH_2-, C^{3-11} alkyl H's), 2.15 (2H, s, -C^2H_2-), 2.35 (2H, t, $J_{C11-C12}$=6.25Hz, -$C^{12}H_2$-), , 3.20 (1H, m, -N^aH-), 4.20 (1H, m, -$C^{15}H_2$-), 4.35 (2H, d, $J_{C15-C14}$=6.25Hz, -$C^{14}H_2$-), 7.25-7.80 (8H, m, aromatic H's).

^{13}C NMR: δ 24.9-34.0 (C^{2-11} alkyl C's), 41.9 (C^{15}), 47.2 (C^{12}), 68.1 (C^{14}), 119.9 – 127.6 (aromatic C's, C^{7-10} + C^{13-19}), 141.3 (C^{13}), 145.3(C^1).

Elemental Analysis; C: 72.18, H: 8.29, N: 2.96 (expected C: 74.47, H: 8.26, N: 3.10).

HPLC-MS ES^+: elution time 18.3 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). Mass peaks; 452.8, MH^+ (100%); 453.8, $M(^{13}C)H^+$ (25%).

7.5 Synthesis of Peptide Sequences

7.5.1 Materials

All amino acids were purchased from Merck Biosciences and used without further purification. 1-(Mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), N-Hydroxybenzotriazole · H_2O (HOBt), Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP). 4-dimethylaminopyridine (DMAP) and diisopropylcarbodiimide (DIC) were purchased from Merck Biosciences. All other reagents were purchased from Sigma-Aldrich.

7.5.2 Resin Loading

Established protocols for estimating the initial resin loading were found to be unsatisfactory so the following protocol was developed.

Samples of the resins were removed and washed with absolute alcohol then dried in vacuum oven at RT for a minimum of 24hrs. 10mg aliquots of the resin were weighed into a 10ml volumetric flask and the volume made up with 20% piperidine in DMF. The samples were left for a minimum of 4 hours with periodic shaking. Readings of the absorbance at 290nm were taken at 20 minute intervals to ensure the deprotection was complete. The equation below was used to determine the approximate loading of the resin.

Loading in mmol per gram = Absorbance / (weight in mg * 0.525)

7.5.3 Deprotection and Monitoring

Fmoc protecting groups were removed with 20% piperidine in DMF for 3 minutes and then 7 minutes using fresh reagents during which time the resin was agitated using N_2 gas. The deprotection was assessed by use of the picryl sulfonic acid test. A few resin beads were removed, washed with DMF and then suspended in fresh DMF. Two drops each of picryl sulfonic acid solution and 10% diisopropylethylamine (DIPEA) in DMF were added and colour allowed to develop for 5 minutes. The solvent was decanted off and the resin washed with DMF. A dark red indicated the presence of free amines and showed the deprotection process was complete. The effectiveness of coupling reactions were also assessed using the picryl sulfonic acid test whereby the lack of red colour showed the completeness of the coupling step.

7.5.4 Protected Peptides

Protected peptide sequences were synthesised using the rink acid labile resin (0.6-0.8mmol /g capacity) with Fmoc protected amino acids.

7.5.4.1 Resin Loading

The resin was swelled in DCM for a minimum of 24hrs prior to use and placed under nitrogen before coupling the first amino acid. Excess DCM was removed from the resin and 5eq of the amino acid, 5eq of MSNT, 3.75 eq of methyl imidazole and DCM (10ml per g of resin) were mixed together under nitrogen and then added to the resin. The coupling was allowed to proceed for 1 hour before the resin was washed with DCM x 3, DMF x 3, DCM x 3 and MeOH x 3. The resin was dried and the resin loading estimated using the procedure above.

7.5.4.2 Chain Elongation

Fmoc deprotections were carried out as per the protocol in section 7.5.3 above. Subsequent amino acids were coupled to the growing sequence using standard PyBOP protocols of 5 eq. of the amino acid, 4.9eq. PyBOP, 5eq. HOBt, 10eq. DIPEA and 10ml DMF per gram of resin. The coupling reaction was allowed to proceed for 30mins upon which time it was tested by the picryl sulfonic acid test described previously. If the test showed partial red colouring the reaction was allowed to proceed for a further 30mins before being tested

again. If the test was still showing partial free amines the resin was washed and the procedure repeated with fresh reagents.

7.5.4.3 Cleavage of Peptide from Resin

The resin was washed with DCM and then 1% TFA in DCM (10ml per g of resin) was added. The mixture was agitated by N2 gas for 2 minutes and the solution was run into 10% pyridine in methanol (2ml per g of resin). This was repeated a further nine times and then the resin was washed with DCM then methanol. The extracts were combined, reduced in volume and precipitated into ice-cold hexane.

7.5.5 Non-Protected and Partially-Protected Peptides

Peptides without protecting groups or with just the 4-bromobenzene sulphonyl protecting group on the arginine were synthesised using a Wang resin with a nominal resin loading of 1.1 mmol/g and Fmoc amino acid protection.

7.5.5.1 Resin Loading

The resin was swelled in DMF for a minimum of 24hrs prior to use and placed under nitrogen before coupling the first amino acid. A symmetrical anhydride was made by dissolving 10eq of the first amino acid in dry DCM, cooling to 0°C and adding 5eq of DIC. The reaction was stirred for 20minutes and then the DCM was removed by rotary evaporation. The symmetrical anhydride was dissolved in DMF (10ml per g of resin) and added to the resin from which excess DMF had been removed. 0.1eq of DMAP was added to the resin and allowed to react for 1 hour.

7.5.5.2 Chain Elongation

Fmoc deprotections were carried out as per the protocol in section 7.5.3 above. Subsequent amino acids were coupled to the growing sequence using standard HBTU protocols of 5 eq. of the amino acid, 5eq. HBTU, 5eq. HOBt, 10eq. DIPEA and 10ml DMF per gram of resin. The coupling reaction was allowed to proceed for 60mins upon which time it was tested by the picryl sulfonic acid test described previously. If the test showed partial red colouring the resin was washed and the procedure repeated with fresh reagents.

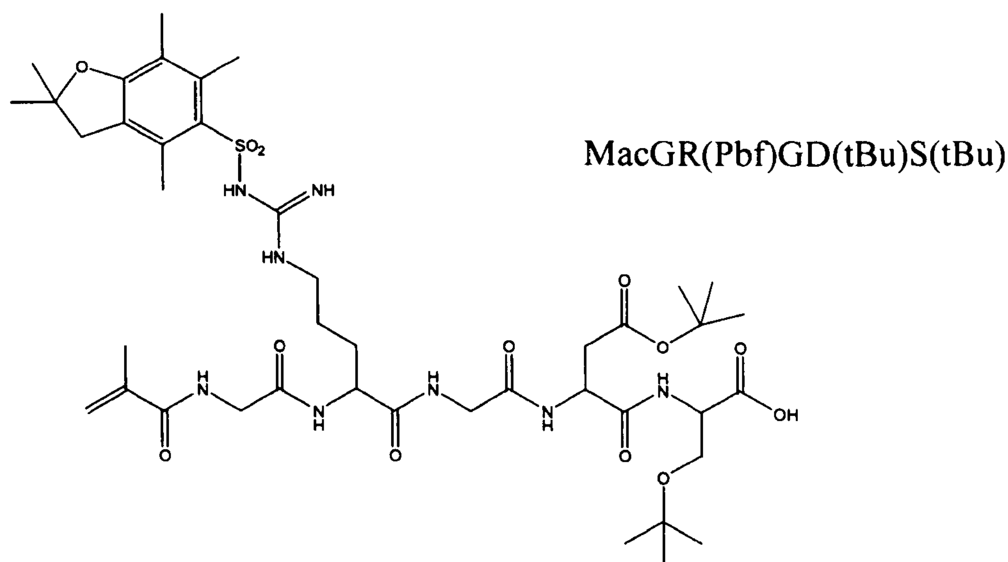
7.5.5.3 Cleavage of Peptide from Resin

The resins were washed with DCM and methanol and dried prior to cleavage to remove all traces of DMF. Peptides were cleaved from the resin using a cleavage cocktail of 95% TFA, 2.5% water and 2.5% triisopropylsilane (TIS) (10 ml per g of resin) for 1 hour. The resin was washed with 1 x cleavage cocktail, 3 x DCM and 3 x ACN. The solvents and TFA were stripped off in a distillation type procedure and the peptide was precipitated into diethyl ether. The products were isolated by centrifugation of the solid/diethyl ether mixture then filtration of the solid followed by re-precipitation from methanol into diethyl ether and centrifugation. The final solid was filtered and dried under vacuum for 24 hours before being stored at -8°C until use.

7.5.6 Analysis and Purification of Peptide Sequences

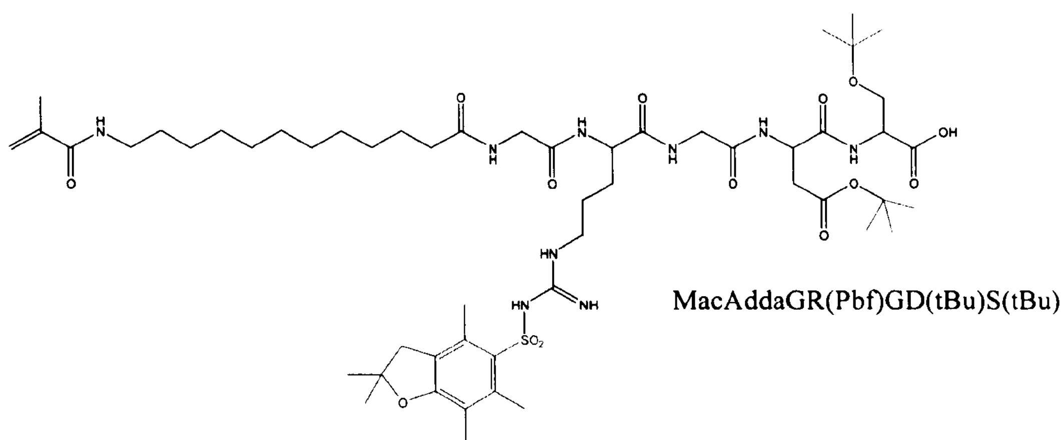
All HPLC analyses were done using a Waters 2690 separation module with a Waters 996 photodiode array detector and an Omnisphere 5 C18 column (250 x 4.6 mm) unless otherwise stated. A gradient of 95% water (containing) 0.1% trifluoroacetic acid): 5% acetonitrile to 100% acetonitrile over 25 minutes was employed. A Micromass Platform LCZ mass spectrometer was used to analyse the traces. Peptides that were determined to have a purity of less than 90% after precipitation from diethyl ether were purified by preparative reverse phase HPLC.

7.5.6.1 Fully protected peptides



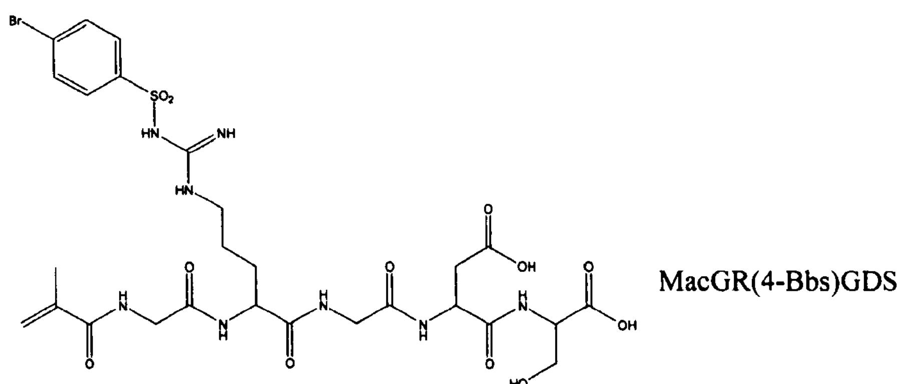
MacGR(Pbf)GD(tBu)S(tBu)

HPLC-MS ES$^+$: elution time 11.81 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). This analysis was performed on a Varian Star HPLC machine. 923.1 MH$^+$ (100%); 924.1 M(^{13}C)H$^+$ (42%). 78% purity by integration of peaks generated by detector at 254nm before preparative HPLC, 91% purity after purification.



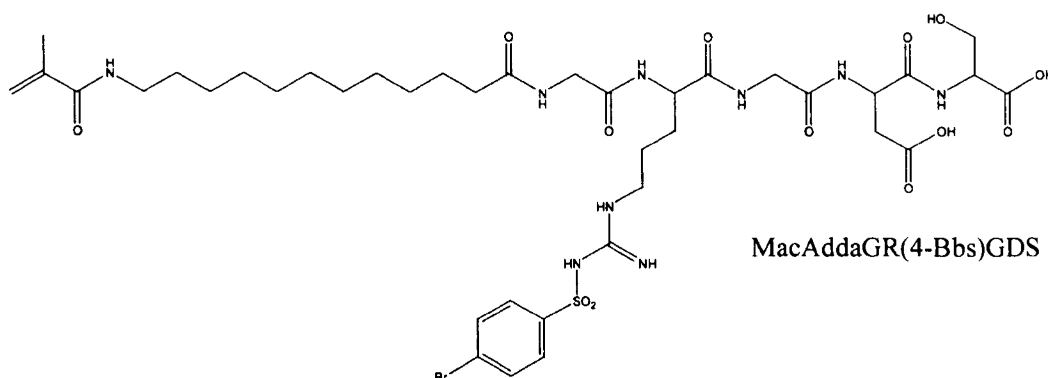
HPLC-MS ES$^+$: elution time 11.81 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). This analysis was performed on a Varian Star HPLC machine. 1120.8 MH$^+$ (100%); 1121.8 M(^{13}C)H$^+$ (55%). 91% purity by integration of peaks generated by detector at 254nm.

7.5.6.2 Partially Protected peptides



MacGR(4-Bbs)GDS: HPLC-MS ES$^+$: elution time 11.65 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). Mass peaks; 781.1, M(^{81}Br^{13}C$_2$)H$^+$ (12%); 780.1, M(^{81}Br^{13}C)H$^+$ (39%); 779.1, M(^{81}Br)H$^+$ (100%); 778.1

$M(^{79}Br^{13}C)H^+$ (31%); 777.1, $M(^{79}Br)H^+$ (92%). 93% purity by integration of peaks generated by diode array.



MacAddaGR(4-Bbs)GDS: HPLC-MS ES^+: elution time 15.67 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). Mass peaks; 978.3, $M(^{81}Br^{13}C_2)H^+$ (17%); 977.3, $M(^{81}Br^{13}C)H^+$ (48%); 976.3, $M(^{81}Br)H^+$ (100%); 975.3 $M(^{79}Br^{13}C)H^+$ (39%); 974.3, $M(^{79}Br)H^+$ (81%). 64% purity by integration of peaks generated by diode array before preparative HPLC, 96% purity after purification.

7.6 Investigation of GST deprotection

7.6.1 Materials

All materials were purchased from Sigma-Aldrich and used without further purification.

7.6.2 Determination of GST Deprotection by Sulphite Assay

Solutions containing 500μM of reduced glutathione (GSH), 0.05-0.5mg/ml glutathione-S-transferase (GST) and 0-500μM of protected arginine derivatives were produced from stock solutions of the relevant compounds and made up to 1ml with 10μmol dm-3 potassium phosphate buffer (PPB, pH 7.4). GSH stock solutions were made up weekly and stored at 3-4°C, GST stock solutions were made up in 5ml batches and frozen in aliquots until use.

The solutions were incubated at the relevant temperatures for a set amount of time upon which they were analysed immediately by the methods detailed below.

An assay for sulphite formation utilising pararosaniline/formaldehyde as used by Zhao et al. and published by Leinweber et al. was also utilised to detect the removal of the protecting group[274,275]. A stock solution of 0.3035g of pararosaniline HCL dye was made up with 10ml of ethanol. 1.6ml of this solution and 4.4ml of concentrated sulphuric acid were combined and made up to 100ml with ultra-pure water. 0.4ml of 40% aqueous formaldehyde was added along with 200mg of activated charcoal to form the assay solution, which was made fresh each day.

500μl of each Z-Arg(4-Bbs) incubation with GST or sulfite standard was added to 200μl of saturated $Hg(II)Cl_{(aq)}$ solution and centrifuged to precipitate the insoluble GSH. 250μl of the supernatant was added to 1ml of the assay solution in a disposable cuvette. The colour was allowed to develop for 15 minutes exactly before being read at 570nm.

Sulphite standards each containing 500μmol dm^{-3} of GSH, of 0, 50, 100, 200, 500 and 1000 μmol dm-3 of sodium sulfite were prepared and formed a linear correlation graph.

<u>7.6.3 Determination of GST Deprotection by HPLC</u>

The enzyme incubation solutions were made up as before (section 7.6.3) except that 0-1000μmol dm^{-3} of a reference amino acid was also included. The reference amino acid was incorporated from a stock solution made up with methanol or acetonitrile.

All HPLC analyses were done using a Waters 2690 separation module with a Waters 996 photodiode array detector and an Omnisphere 5 C18 column (250 x 4.6mm). A gradient of 95% water (containing) 0.1% trifluoroacetic acid): 5% acetonitrile to 100% acetonitrile over 25 minutes was employed with a 25min downwards gradient included where enzyme was present to wash the enzyme from the column. The amino acids were detected using a photodiode array from 200 to 320nm. The concentration of the arginine derivative amino acid was determined by integrating the peak areas and using the ratio of peak areas to determine the concentration from a calibration chart. The calibration charts were formulated using 0, 20, 40, 60, 80, 100, 200, 300, 400 and 500μM solutions of the amino acid with 50, 100, or 1000μM of the reference amino acid and 500mmol dm^{-3} of GSH.

7.7 Peptide-Containing Hydrogel Synthesis

7.7.1 Synthesis of polymers

Stock solutions of the peptides were made with methanol to a concentration of 10 picomoles / ml. These stock solutions were diluted with methanol to form solutions with concentrations of 1, 0.1 and 0.01pmol/ml. Stock solutions of monomers, cross-linker and 1 wt% initiator were made up and stored at -8°C in the dark until use. Peptide and monomer stock solutions were volumetrically measured (volumes adjusted for density) and combined immediately prior to use. Thermally cured polymer sheets were synthesised as described in section 5.3.3 and UV cured polymer thin-films were synthesised as described in section 5.1.2.

7.7.2 Enzyme Deprotection of Peptides

Disks of polymers were cut out and placed into 24 well plates in a sterile culture hood. The disks were washed three times with ethanol and three times with PBS. A stock solution of GST 1mg/ml and GSH 10mmol dm^{-3} in PBS was made up and then filter sterilised. The stock solution was diluted by a factor of 20 with sterile PBS resulting in a final concentration of GST 0.05mg/ml and GSH 500 μM. The final enzyme-containing solution was added to wells containing polymers (0.5 ml/well) and these were placed in an incubator at 37°C and 5% CO_2 for a set time period. After the enzyme incubations were complete the polymers were washed three times with PBS and twice with serum free media prior to cell culture.

7.8 Cell Culture

All materials were obtained from Sigma-Aldrich UK. Deionised water was used throughout. Dulbecco's modified eagles media (DMEM) supplemented with $2x10^{-3}$ mol L^{-1} glutamine, 0.625 μg ml^{-1} amphotercin B, 100 i.u. ml penicillin and 100 μg ml^{-1} streptomycin was routinely utilised for all experiments. In addition where serum containing media was utilised 10% v/v fetal calf serum was added.

7.8.1 Cell Maintenance

Fibroblasts were obtained from human skin obtained from patients undergoing elective surgery who gave informed consent for skin not required for their treatment to be used for research (as detailed in reference[276]). Skin was placed in 0.1% trypsin w/v overnight before the epidermis was peeled from the dermis. This dermis was washed in phosphate-buffered saline (pH 7.2), finely minced and then placed in 0.5% collagenase-A at 37°C for 16 h. Cells were then cultured in DMEM media supplemented with 10% v/v neonatal calf serum, 2×10^{-3} mol L^{-1} glutamine, 0.625 mg ml^{-1} amphotercin B, 100 i.u. ml penicillin and 100 mg ml^{-1} streptomycin. Cells were passaged routinely using 0.02% (w/v) EDTA and used for culture on polymers between passage numbers 5 and 12.

7.8.2 Cell Culture on Polymers

Cells were harvested at ~90% confluency by incubating with trypsin until cells were detached. Serum-containing media was added to neutralise the trypsin and the cell suspension was centrifuged and the media containing trypsin was decanted. After harvesting cells were suspended in the appropriate media (either serum free or containing 10% FCS). The concentration of cells was determined by mixing 20μL of the cell suspension with 20μL of tryphan blue and counting the number of live cells in a known volume using a haemocytometer. The cell suspension was diluted to give an appropriate volume that would contain 100,000 cells (20-40μL). This volume was applied to each well containing polymer or control substances and the cells were allowed to adhere for 20 minutes. After this time 1 ml of the appropriate media was applied to the wells and the cells were incubated for a set time period after which time the cells were photographed through a phase contrast microscope and/or analysed via the MTT assay.

7.8.3 Competition with Soluble RGD

A solution of 1 mg/ml GRGDS in serum-free media was prepared. After 24 hours of cell culture as described above the media was removed from all wells. 0.5ml of GRGDS containing media was added to half of the wells and 0.5 ml of serum free media was applied to the other half of the wells as a control. Photographs of the cells were taken at 0, 15, 30, 45 and 60 minutes after the application of the GRGDS-containing media through a phase contrast

microscope. After 60 minutes the cells were analysed by the MTT assay as described below.

7.8.4 Incubation with Non-Contacting Cells

Cells were harvested at ~90% confluency by incubating with trypsin until cells were detached. Serum-containing media was added to neutralise the trypsin and the cell suspension was centrifuged and the media containing trypsin was decanted. After harvesting cells were suspended in serum free media. The concentration of cells was determined by mixing 20μL of the cell suspension with 20μL of tryphan blue and counting the number of live cells in a known volume using a haemocytometer. The cell suspension was diluted to give an appropriate volume that would contain 100,000 cells (20-40μL). This volume was applied to a co-culture basket suspended in a well above a polymer or control substances and the cells were allowed to adhere for 20 minutes. After this time 1 ml of the appropriate media was applied to the wells and the cells were incubated for a set time. After this time the co-culture baskets were removed and the polymers were sterilised with ethanol to kill any cells that had migrated onto the surface. The surfaces were washed x 3 with ethanol and then normal cell culture was performed on the polymers as per section 7.8.2.

7.8.5 MTT Assay

Cells were washed with PBS and then 1 ml of MTT solution was added (0.5 mg MTT / ml PBS) to each well. The cells were incubated at 37°C and 5% CO_2 for 40 minutes. The MTT solution was removed and 300μL of ethoxy ethanol was added to each well to solubilise the formazan colour formed by the assay. 150μL of ethoxy ethanol was removed from each well and placed in a 96 well plate. The absorbance at 540 nm was read using a Dynatech MR5000 plate reader with a reference wavelength of 630nm.

7.8.6 Histology

After a set time point (30mins, 1,2,4 or 24 hours) cells were washed with PBS and then fixed for 10 minutes with 10% formalin in PBS. The cells were washed with PBS x 3 and then 0.1% triton was added for 10 minutes to permeabilise the cell membranes. The cells were again washed with PBS x 3 and

then 250μl of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (300nM in PBS) was added for 20 minutes. Following a further 3 PBS washes a solution of FITC-phalloidin (0.5μg/ml in PBS containing 1% methanol) was added for 5 minutes. After a further 3 PBS washes the cells were observed visually using epi-fluorescence microscopy using an ImageXpress® automated cellular imaging and analysis system (DAPI λ_{ex} = 358 nm, λ_{em} = 461 m; FITC λ_{ex} = 490 nm, λ_{em} = 523 nm; Axon Instruments, CA, USA).

8 References

1. Freshney, R. I. *Culture of Animal Cells: A Manual of Basic Techniques* (Wiley-Liss, 1994).
2. Kleinsmith, L. J. & Kish, V. M. *Principles of Cell & Molecular Biology* (Harper Collins College Publishers, 1995).
3. Seal, B. L., Otero, T. C. & Panitch, A. Polymeric biomaterials for tissue and organ regeneration. *Materials Science & Engineering* **R34**, 147-230 (2001).
4. Lebaron, R. G. & Kyriacos, A. A. Extracellular Matrix Cell Adhesion Peptides: Functional Applications in Orthopedia Materials. *Tissue Engineering* **6**, 85-103 (2000).
5. Shin, H., Jo, S. & Mikos, A. G. Biomimetic materials for tissue engineering. *Biomaterials* **24**, 4353-4364 (2003).
6. Li, F., Griffith, M., Li, Z., Tanodekaew, S., Sheardown, H., Hakim, M. & Carlsson, D. J. Recruitment of multiple cell line by collagen-synthetic copolymer matrices in corneal regeneration. *Biomaterials* **26**, 3093-3104 (2005).
7. Babensee, J. E., anderson, J. M., McIntire, L. V. & Mikos, A. G. Host response to tissue engineered devices. *Advanced Drug Delivery Reviews* **33**, 111-139 (1998).
8. Ma, P. X. Scaffolds for tissue fabrication. *Materials Today* **May 2004**, 30-40 (2004).
9. Drury, J. L. & Mooney, D. J. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* **24**, 4337-4351 (2003).
10. Ramakrishna, S., Mayer, J., Wintermantel, E. & Leong, K. W. Biomedical applications of polymer-composite materials: a review. *Composites Science and Technology* **61**, 1189-1224 (2001).
11. Baroli, B. Photopolymerization of biomaterials: issues and potentialities in drug delivery, tissue engineering, and cell encapsulation applications. *Journal of Chemical Technology and Biotechnology* **81**, 491-499 (2006).
12. Peter, S. J., Miller, M. J., Yasko, A. W., Yaszemski, M. J. & Mikos, A. G. Polymer Concepts in Tissue Engineering. *Applied Biomaterials* **43**, 422-427 (1998).
13. Hutmacher, D. W. Scaffolds in tissue engineering bone and cartilage. *Biomaterials* **2000**, 2529 (2000).
14. Tziampazis, E., Kohn, J. & Moghe, P. V. PEG-variant Biomaterials as Selectively Adhesive Protein Templates: Model Surfaces for Controlled Cell Adhesion and Migration. *Biomaterials* **21**, 511-520 (2000).
15. Burmeister, J. S., McKinney, V. Z., Reichert, W. M. & Truskey, G. A. Role of Endothelial Cell-Substrate Contact Area and Fibronectin-Receptor Affinity in Cell Adhesion to HEMA/EMA Copolymers. *Journal of Biomedical Materials Research* **47**, 577-584 (1999).
16. Dewez, J. L., Doren, A., Schneider, Y. J. & Rouxhet, P. G. Competitive Adsorption of Proteins: Key of the Relationship Between Substratum Surface Properties and Adhesion of Epithelial Cells. *Biomaterials* **20**, 547-559 (1999).
17. Haigh, R., Rimmer, S. & Fullwood, N. J. Synthesis and Properties of Amphiphilic Networks 1: The Effect of Hydration and Polymer Composition on the Adhesion of Immunoglobin-G to Poly(Laurylmethacrylate-stat-Glycerolmonomethacrylate-stat-ethylene-glycol-dimethacrylate) Networks. *Biomaterials* **21**, 736-739 (2000).

18. Ryang-Kim, M., Jeong, J. H. & Park, T. G. Swelling Induced Detachment of Chrondrocytes Usig RGD-Modified Poly(N-isopropylacrylamide) Hydrogel Beads. *Biotechnol. Prog.* **18**, 495-500 (2002).
19. Beer, J. H., Springer, K. T. & Coller, B. S. Immobilized Arg-Gly-Asp (RGD) Peptides of Varying Lengths as Structural Probes of the Platelet Glycoprotein IIb / IIIa Receptor. *Blood* **79**, 117-128 (1992).
20. Kannan, R. Y., Salacinski, H., Sales, K., Butler, P. & Seifalian, A. M. The roles of tissue engineering and vascularisation in the development of micro-vascular networks: a review. *Biomaterials* **26**, 1857-1875 (2005).
21. Leong, K. F., Cheah, C. M. & Chua, C. K. Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs. *Biomaterials* **24**, 2363-2378 (2003).
22. Tsang, V. L. & Bhatia, S. N. Three-dimensional tissue fabrication. *Advanced Drug Delivery Reviews* **56**, 1635-1647 (2004).
23. Ho, M.-H., Wang, D.-A., Hsieh, H.-J., Liu, H.-C., Hsien, T.-Y., Lai, J.-Y. & Hou, L.-T. Preparation and characterization of RGD-immobilized chitosan scaffolds. *Biomaterials* **26**, 3197-3206 (2005).
24. Hsu, S.-H., Chang, S.-H., Yen, H.-J., Whu, S. W., Tsai, C.-L. & Chen, D. C. Evaluation of Biodegradable Polymers Modified by Type II Collagen and Arg-Gly-Asp as Tissue Engineering Scaffolding Materials for Cartilage Regeneration. *Artificial Organs* **30**, 42-55 (2006).
25. Yoon, J. J., Song, S. H., Lee, D. S. & Park, T. G. Immobilization of cell adhesive RGDpeptide onto the surface of highly porous biodegradable polymer scaffolds fabricated by a gas foaming/salt leaching method. *Biomaterials* **25**, 5613-5620 (2004).
26. Kim, T. G. & Park, T. G. Biomimicking Extracellular Matrix: Cell adhesive RGD Peptide Modified Electrospun Poly(D,L-lactic-co-glycolic acid) Nanofiber Mesh. *Tissue Engineering* **12**, 221-233 (2006).
27. Schense, J. C. & Hubbell, J. A. Three-dimensional Migration of Neurites Is Mediated by Adhesion Site Density and Affinity. *Journal of Biological Chemistry* **275**, 6813-6818 (2000).
28. Burgess, B. T., Myles, J. L. & Dickinson, R. B. Quantitative Analysis of Adhesion-Mediated Cell Migration in Three-Dimensional Gels of RGD-Grafted Collagen. *Annals of Biomedical Engineering* **28**, 110-118 (2000).
29. Fisher, J. P., Dean, D., Engel, P. S. & Mikos, A. G. Photoinitiated Polymerization of Biomaterials. *Annu. Rev. Mater. Res.* **31**, 171-181 (2001).
30. Mann, B. K., Gobin, A. S., Tsai, A. T., Schmedlen, R. H. & West, J. L. Smooth Muscle Cell Growth in Photopolymerised Hydrogels with Cell Adhesive and Proteolytically Degradable Domains: Synthetic ECM Analogs for Tissue Engineering. *Biomaterials* **22**, 3045-3051 (2001).
31. Burdick, J. A. & Anseth, K. S. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. *Biomaterials* **23**, 4315-4323 (2002).
32. Park, K.-H., Na, K., Kim, S. W., Jung, S. Y., Park, K. H. & Chung, H.-M. Phenotype of hepatocyte spheroids behavior within thermo-sensitive poly(NiPAAm-co-PEG-g-GRGDS) hydrogel as a cell delivery vehicle. *Biotechnology Letters* **27**, 1081-1086 (2005).
33. Park, K.-H., Na, K., Jung, S. Y., Kim, S. W., Park, K. H., Cha, K. Y. & Chung, H.-M. Insulinoma Cell Line (M1N6) Adhesion and Spreading Mediated by Arg-Gly-Asp (RGD) Sequence Conjugated in Thermo-Reversible Gel. *Journal of Bioscience and Bioengineering* **99**, 598-602 (2005).

34. Yang, F., Willams, C. G., Wang, D.-A., Lee, H., Manson, P. N. & Elisseeff, J. H. The effect of incorporating RGD adhesive peptide in polyethylene glycol diacrylate hydrogel on osteogenesis of bone marrow stromal cells. *Biomaterials* **26**, 5991-5998 (2005).
35. Schense, J. C. Cross-Linking Exogenous Bifunctional Peptides into Fibrin Gels with Factor XIIIa. *Bioconjugate Chemistry* **10**, 75-81 (1999).
36. Alsberg, E., Anderson, K. W., Albeiruti, A., Rowley, J. A. & Mooney, D. J. Engineering growing tissues. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 12025-12030 (2001).
37. Nguyen, K. T. & West, J. L. Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials* **23**, 4307-4314 (2002).
38. Baroli, B. Review: Photopolymerization of biomaterials: issue and potentialities in drug delivery, tissue engineering, and cell encapsulation applications. *Journal of Chemical Technology and Biotechnology* **81**, 491-499 (2006).
39. Hubbell, J. A. Bioactive biomaterials. *Current Opinion in Biotechnology* **10**, 123-129 (1999).
40. Drumheller, P. D. & Hubbell, J. A. Polymer Networks with Grafted Cell Adhesion Peptides for Highly Biospecific Cell Adhesive Substrates. *Analytical Biochemistry* **222**, 380-388 (1994).
41. Hern, D. L. & Hubbell, J. A. Incorporation of Adhesion Peptides into Nonadhesive Hydrogels Useful for Tissue Resurfacing. *Journal of Biomedical Materials Research* **39**, 266-276 (1998).
42. Danilov, Y. N. & Juliano, R. L. $(Arg\text{-}Gly\text{-}Asp)_n$-Albumin Conjugates as a Model Substratum for Integrin-Mediated Cell Adhesion. *Experimental Cell Research* **182**, 186-196 (1989).
43. Delforge, D., Gillon, B., Art, M., Dewelle, J., Raes, M. & Remacle, J. Design of a synthetic adhesion protein by grafting RGD tailed cyclic peptides on bovine serum albumin. *Letters in Peptide Science* **5**, 87-91 (1998).
44. Sharma, R. I., Pereira, M., Schwarzbauer, J. E. & Moghe, P. V. Albumin-derived nanocarriers: Substrates for enhanced cell adhesive ligand display and cell motility. *Biomaterials* **27**, 3589-3598 (2006).
45. Lee, K. Y., Alsberg, E., Hsiong, S., Comisar, W., Linderman, J., Ziff, R. & Mooney, D. J. Nanoscale Adhesion Ligand Organization Regulates Osteoblast Proliferation and Differentiation. *Nano Letters* **4**, 1501-1506 (2004).
46. Rowley, J. A., Madlambayan, G. & Mooney, D. J. Alginate Hydrogels as Synthetic Extracellular Matrix Materials. *Biomaterials* **20**, 45-53 (1999).
47. Marler, J. J., Guham, A., Rowley, J., Koka, R., Mooney, D., Upton, J. & Vacanti, J. Soft-Tissue Augmentation with Injectable Alginate and Syngeneic Fibroblasts. *Plastic & Reconstructive Surgery* **105**, 2049-2058 (2000).
48. Bouhadir, K. H., Hausman, D. S. & Mooney, D. J. Synthesis of cross-linked poly(aldehyde guluronate) hydrogels. *Polymer* **40**, 3575-3584 (1999).
49. de Taillac, L. B., Porte-Durrieu, M. C., Labrugere, C., Bareille, R., Amedee, J. & Baquey, C. Grafting of RGD peptides to cellulose to enhance human osteoprogenitor cells adhesion and proliferation. *Composites Science and Technology* **64**, 827-837 (2004).
50. Chung, T.-W., Lu, Y.-F., Wang, H.-Y., Chen, W.-P., Wang, S.-S., Lin, Y.-S. & Chu, S.-H. Growth of Human Endothelial Cells on Different Concentrations of Gly-Arg-Gly-Asp Grafted Chitosan Surface. *Artificial Organs* **27**, 155-161 (2003).

51. Masuko, T., Iwasaki, N., Yamane, S., Funakoshi, T., Majima, T., Minami, A., Ohsuga, N., Ohta, T. & Nishimura, S.-I. Chitosan–RGDSGGC conjugate as a scaffold material for musculoskeletal tissue engineering. *Biomaterials* **26**, 5339-5347 (2005).
52. Yamauchi, K., Hojo, H., Yamamoto, Y. & Tanabe, T. Enhanced cell adhesion on RGDS-carrying keratin film. *Materials Science & Engineering C* **23**, 467-472 (2003).
53. McDevitt, T., Nelson, K. E. & Stayton, P. S. Constrained Cell Recognition Peptides Engineering into Streptavidin. *Biotechnol. Prog.* **15**, 391-396 (1999).
54. Gümüsderelioglu, M. & Turkoglu, H. Biomodification of non-woven polyester fabrics by insulin and RGD for use in serum-free cultivation of tissue cells. *Biomaterials* **23**, 3927-3935 (2002).
55. Quirk, R. A., Davies, M. C., Tendler, S. J. B., Chan, W. C. & Shakesheff, K. M. Controlling Biological Interactions with Poly(lactic acid) by Surface Entrapment Modification. *Langmuir* **17**, 2817-2820 (2001).
56. Yang, X. B., Roach, H. I., Clarke, N. M. P., Howdle, S. M., Quirk, R., Shakesheff, K. M. & Oreffo, R. O. C. Human Osteoprogenitor Growth and Differentiation on Synthetic Biodegradable Structures After Surface Modification. *Bone* **29**, 523-531 (2001).
57. Marletta, G., Ciapetti, G., Satriano, C., Pagani, S. & Baldini, N. The effect of irradiation modification and RGD sequence adsorption on the response of human osteoblasts to polycaprolactone. *Biomaterials* **26**, 4793-4804 (2005).
58. Mizutani, M., Arnold, S. C. & Matsuda, T. Liquid, Phenylazide-End-Capped Copolymers of E-Caprolactone and Trimethylene Carbonate: Preparation, Photocuring Characteristics, and Surface Layering. *Biomacromolecules* **3**, 668-675 (2002).
59. Santiago, L. Y., Nowak, R. W., Rubin, J. P. & Marra, K. G. Peptide-surface modification of poly(caprolactone) with laminin-derived sequences for adipose-derived stem cell applications. *Biomaterials* **27**, 2962-2969 (2006).
60. Quirk, R. A., Chan, W. C., Davies, M. C., Tendler, S. J. B. & Shakesheff, K. M. Poly(L-Lysine)-GRGDS as a Biomimetic Surface Modifier for Poly(Lactic Acid). *Biomaterials* **22**, 865-872 (2001).
61. Lin, H.-B., Garcia-Echeverria, C., Asakura, S., Sun, W., Mosher, D. F. & Cooper, S. L. Endothelial cell adhesion on polyurethanes containing covalently attached RGD-peptides. *Biomaterials* **13**, 905-914 (1992).
62. Sidouni, F.-Z., Nurdin, N., Chabrecek, P., Lohmann, D., Vogt, J., Xanthopoulos, N., Mathieu, H. J., Francois, P., Vaudaux, P. & Descouts, P. Surface properties of a specifically modified high-grade medical polyurethane. *Surface Science* **491**, 355-369 (2001).
63. Wang, D.-A., Ji, J., Sun, Y.-H., Shen, J.-C., Feng, L.-X. & Elisseeff, J. H. In Situ Immobilization of Proteins and RGD Peptide on Polyurethane Surfaces via Poly(ethylene oxide) Coupling Polymers for Human Endothelial Cell Growth. *Biomacromolecules* **3**, 1286-1295 (2002).
64. Anderheiden, D., Klee, D., Hocker, H., Heller, B., Kirkpatrick, C. J. & Mittermayer, C. Surface modification of a biocompatible polymer based on polyurethane for artificial blood vessels. *Journal of Materials Science: Materials in Medicine* **3**, 1-4 (1992).
65. Krijgsman, B., Seifalian, A. M., Salacinski, H., Tai, N. R., Punshon, G., Fuller, B. J. & Hamilton, G. An Assessment of Covalent Grafting of RGD Peptides to the Surface of a Compliant Poly(Carbonate-Urea)Urethane Vascular

Conduit versus Conventional Biological Coatings: Its Role in Enhancing Cellular Retention. *Tissue Engineering* **8**, 673-680 (2002).
66. Bruers, W., Klee, D., Hocker, H. & Mittermayer, C. Immobilization of a fibronectin fragment at the surface of a polyetherurethane film. *Journal of Materials Science: Materials in Medicine* **2**, 106-109 (1991).
67. Lopez, L. C., Gristina, R., Ceccone, G., Rossi, F., Favia, P. & d'Agostino, R. Immobilization of RGD peptides on stable plasma-deposited acrylic acid coatings for biomedical devices. *Surface& Coating Technology* **200**, 1000-1004 (2005).
68. Brandley, B. K. & Schnaar, R. L. Covalent Attachment of an Arg-Gly-Asp Sequence Peptide to Derivatizable Polyacrylamide Surfaces: Support of Fibroblast Adhesion and Long-Term Growth. *Analytical Biochemistry* **172**, 270-278 (1988).
69. Brandley, B. K. & Schnaar, R. L. Tumor Cell Haptotaxis on Covalently Immobilized Linear and Exponential Gradients of a Cell Adhesion Peptide. *Developmental Biology* **135**, 74-86 (1989).
70. Brandley, B. K., Schnaar, R. L. & Shaper, J. H. Tumor Cell Haptotaxis on Immobilized N-Acteylglucosamine Gradients. *Developmental Biology* **140**, 167-171 (1990).
71. Weisz, O. A. & Schnaar, R. L. Hepatocyte Adhesion to Carbohydrate-Derivatized Surfaces II. Regulation of Cytoskeletal Organization and Cell Morphology. *Journal of Cell Biology* **115**, 495-504 (1991).
72. Willcox, P. J., Reinhart-King, C. A., Lahr, S. J., DeGrado, W. F. & Hammer, D. A. Dynamic heterodimer-functionalized surfaces for endothelial cell adhesion. *Biomaterials* **26**, 4757-4766 (2005).
73. Smith, E., Yang, J., McGann, L., Sebald, W. & Uludag, H. RGD-grafted thermoreversible polymers to facilitate attachment of BMP-2 responsive C2C12 cells. *Biomaterials* **26**, 7329-7338 (2005).
74. Stile, R. A. & Healy, K. E. Thermo-responsive Peptide Modified Hydrogels for Tissue Regeneration. *Biomacromolecules* **2**, 185-194 (2001).
75. Na, K. & Park, K.-H. Conjugation of Arg-Gly-Asp sequence into thermo-reversible gel as an extracellular matrix for 3T3-L1 fibroblast cell culture. *Biotechnology Letters* **22**, 1553-1556 (2000).
76. Ebara, M., Yamato, M., Aoyagi, T., Kikuchi, A., Sakai, K. & Okano, T. Temperature-Responsive Cell Culture Surfaces Enable "On-Off" Affinity Control between Cell Integrins and RGDS Ligands. *Biomacromolecules* **5** (2004).
77. Kantlehner, M., Finsinger, D., Meyer, J., Schaffner, P., Jonczyk, A., Diefenbach, B., Nies, B. & Kessler, H. Selective RGD-Mediated Adhesion of Osteoblasts at Surfaces of Implants. *Angew. Chem. Int. Ed.* **38**, 560-562 (1999).
78. Koo, L. Y., Irvine, D. J., Mayes, A. M., Lauffenburger, D. A. & Griffith, L. G. Co-regulation of Cell Adhesion by Nanoscale RGD Organization and Mechanical Stimulus. *Journal of Cell Science* **115**, 1423-1433 (2002).
79. Irvine, D. J. & Mayes, A. M. Nanoscale Clustering of RGD Peptides at Surfaces Using Comb Polymers 1. Synthesis and Characterisation of Comb Thin Films. *Biomacromolecules* **2**, 85-94 (2001).
80. Patel, S., Thakar, R. G., Wong, J., McLeod, S. D. & Li, S. Control of cell adhesion on poly(methyl methacrylate). *Biomaterials* **27**, 2890-2897 (2006).
81. Schneider, G. B., English, A., Abraham, M., Zaharias, R., Stanford, C. & Keller, J. The effect of hydrogel charge density on cell attachment. *Biomaterials* **25**, 3023-3028 (2004).

82. Jacob, J. T., Rochefort, J. R., Bi, J. & Gebhart, B. M. Corneal Epithelial Cell Growth Over Tethered-Protein/Peptide Surface-Modified Hydrogels. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **72B**, 198-205 (2004).
83. Woerly, S., Pinet, E., Robertis, L. d., Diep, D. V. & Bousmina, M. Spinal Cord Repair with PHPMA Hydrogel Containing RGD Peptides (NeuroGel^{TM}). *Biomaterials* **22**, 1095-1111 (2001).
84. Loh, N. K., Woerly, S., Bunt, S. M., Wilton, S. D. & R., H. A. The Regrowth of Axons within Tissue Defects in the CNS Is Promoted by Implanted Hydrogel Matrices That Contain BDNF and CNTF Producing Fibroblasts. *Experimental Neurology* **170**, 72-84 (2001).
85. Plant, G. W., Woerly, S. & Alan R., H. Hydrogels Containing Peptide or Aminosugar Sequences Implanted into the Rat Brain: Influence on Cellular Migration and Axonal Growth. *Experimental Neurology* **143**, 287-299 (1997).
86. Schmedlen, R. H., Masters, K. S. & West, J. L. Photocrosslinkable Polyvinyl Alcohol Hydrogels that can be Modified with Cell Adhesion Peptides for use in Tissue Engineering. *Biomaterials* **23**, 4325-4332 (2002).
87. Gümüsderelioglu, M., Muftuoglu, O. & Karakecili, A. G. Biomodification of thermosensitive copolymer of ethylene glycol vinyl ether by RGD and insulin. *Reactive & Functional Polymers* **58**, 149-156 (2004).
88. Ijama, H. & Kawakami, K. Promotion of Monolayer Formation and High Expression of Ammonia Metabolism of Primary Rat Hepatocytes on Arginine-Glycine-Aspartic Acid-Containing Peptide-Coated Polystyrene Dish. *Journal of Bioscience and Bioengineering* **100**, 62-66 (2005).
89. Kurihara, H. & Nagamune, T. Cell Adhesion Ability of Artificial Extracellular Matrix Proteins Containing a Long Repetitive Arg-Gly-Asp Sequence. *Journal of Bioscience and Bioengineering* **100**, 82-87 (2005).
90. Rapuano, B. E., Wu, C. & MacDonald, D. E. Osteoblast-like cell adhesion to bone sialoprotein peptides. *journal of Orthopaedic Research* **22**, 353-361 (2004).
91. Varani, J., Inman, D. R., Fligiel, S. E. G. & Hillegas, W. J. Use of recombinant and synthetic peptide as attachment factors for cells on microcarriers. *Cytotechnology* **13**, 89-98 (1993).
92. Verrier, S., Pallu, S., Bareille, R., Jonczyk, A., Meyer, J., Dard, M. & Amedee, J. Function of linear and cyclic RGD-containing peptides in osteoprogenitor cells adhesion process. *Biomaterials* **23**, 585-596 (2002).
93. Bhadriraju, K. & Hansen, L. K. Hepatocyte adhesion, growth and differentiated function on RGD-containing proteins. *Biomaterials* **21**, 267-272 (2000).
94. DeLong, S. A., Moon, J. J. & West, J. L. Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration. *Biomaterials* **26**, 3227-3234 (2005).
95. Drumheller, P. D., Elbert, D. L. & Hubbell, J. A. Multifunctional Poly(ethylene glycol) Semi-interpenetrating Polymer Networks as Highly Selective Adhesive Substrates for Bioadhesive Peptide Grafting. *Biotech. Bioeng.* **43**, 772-780 (1994).
96. Elbert, D. L. & Hubbell, J. A. Conjugate Addition Reactions Combined with Free-Radical Cross-Linking for the Design of Materials for Tissue Engineering. *Biomacromolecules* **2**, 430-441 (2001).
97. Faraasen, S., Voros, J., Csucs, G., Textor, M., Merkle, H. P. & Walter, E. Ligand-Specific Targeting of Microspheres to Phagocytes by Surface

Modification with Poly(L-Lysine)-Grafter Poly(Ethylene Glycol) Conjugate. *Pharmaceutical Research* **20**, 237-246 (2003).
98. Gobin, A. S. & West, J. L. Cell migration through defined, synthetic extracellular matrix analogues. *FASEB Journal* **online** (2002).
99. Halstenberg, S., Panitch, A., Rizzi, S., Hall, H. & Hubbell, J. A. Biologically Engineered Protein-*graft*-Poly(ethylene glycol) Hydrogels: A Cell Adhesive and Plasmin Degradable Biosynthetic Material for Tissue Repair. *Biomacromolecules* **3**, 710-723 (2002).
100. Jo, S., Shin, H. & Mikos, A. G. Modification of Oligo(poly(ethylene glycol)fumarate) Macromer with a GRGD Peptide for the Preparation of Functionalised Polymer Networks. *Biomacromolecules* **2**, 255-261 (2001).
101. Kao, W. J. & Hubbell, J. A. Murine Macrophage Behaviour on Peptide-Grafted Polyethyleneglycol-Containing Networks. *Biotech. Bioeng.* **59**, 2-9 (1998).
102. Kao, W. J., Hubbell, J. A. & anderson, J. M. Protein-mediated macrophage adhesion and activation on biomaterials: a model for modulating cell behaviour. *Journal of Materials Science: Materials in Medicine* **10**, 601-605 (1999).
103. Kao, W. J., Lee, D., Schense, J. C. & hubbell, J. A. Fibronectin modulates macrophage adhesion and FBGC formation: The role of RGD, PHSRN, and PRRARV domains. *Journal of Biomedical Materials Research* **55**, 79-88 (2001).
104. Kao, W. J. & Lee, D. In vivo modulation of host response and macrophage behavior by polymer networks grafted with fibronectin-derived biomimetic oligopeptides: the role of RGD and PHSRN domains. *Biomaterials* **22**, 2901-2909 (2001).
105. Lieb, E., Hacker, M., Tessmar, J., Kunz-Schughart, L. A., Fiedler, J., Dahmen, C., Hersel, U., Kessler, H., Schulz, M. B. & Gopferich, A. Mediating specific cell adhesion to low-adhesive diblock copolymers by instant modification with cyclic RGD peptides. *Biomaterials* **26**, 2333-2341 (2005).
106. Lin, Y.-S., Wang, S.-S., Chung, T.-W. & Wang, Y.-H. Growth of Endothelial Cells on Different Concentrations of Gly-Arg-Gly-Asp Photochemically Grafted in Polyethylene Glycol Modified Polyurethane. *Artificial Organs* **25**, 617-621 (2001).
107. Liu, Y. & Kao, W. J. Human macrophage adhesion on fibronectin: The role of substratum and intracellular signalling kinases. *Cellular Signalling* **14**, 145-152 (2002).
108. Mann, B. K., Schmedlen, R. H. & West, J. L. Tethered-TGF-B increases Extracellular Matrix Production of Vascular Smooth Muscle Cells. *Biomaterials* **22**, 439-444 (2001).
109. Nuttelman, C. R., Tripodi, M. C. & Anseth, K. S. Synthetic hydrogel niches that promote hMSC viability. *Matrix Biology* **24**, 208-218 (2005).
110. Ochsenhirt, S. E., Kokkoli, E., McCarthy, J. B. & Tirrell, M. Effect of RGD secondary structure and the synergy site PHSRN on cell adhesion, spreading and specific integrin engagement. *Biomaterials* **27**, 3863-3874 (2006).
111. Park, Y. D., Tirelli, N. & Hubbell, J. A. Photopolymerized halouronic acid-based hydrogels and interpentrating networks. *Biomaterials* **24**, 893-900 (2003).
112. Phillips, J. M. & Kao, W. J. Macrophage Adhesion on Gelatin-Based Interpenetrating Networks Grafted with PEGylated RGD. *Tissue Engineering* **11**, 964-973 (2005).

113. Schuler, M., Owen, G. R., Hamilton, D. W., de Wild, M., Textor, M., Brunette, D. M. & Tosatti, S. G. P. Biomimetic modification of titanium dental implant model surfaces using the RGDSP-peptide sequence: A cell morphology study. *Biomaterials* **27**, 4003-4015 (2006).
114. Neff, J. A., Tresco, P. A. & Caldwell, K. D. Surface Modification for the Controlled Studies of Cell-Ligand Interactions. *Biomaterials* **20**, 2377-2393 (1999).
115. Zhu, J., Beamish, J. A., Tang, C., Kottke-Marchant, K. & Marchant, R. E. Extracellular Matrix-like Cell-Adhesive Hydrogels from RGD-Containing Poly(ethylene glycol) Diacrylate. *Macromolecules* **39**, 1305-1307 (2006).
116. Zhao, B., Tian, W., Feng, H., Lee, I.-S. & Cui, F. Effects of RGD peptide grafting to titanium dental implants on the adhesion of human gingival fibroblasts and epithelial cells. *Current Applied Physics* **5**, 407-410 (2005).
117. Xiao, S.-J., Textor, M. & Spencer, N. D. Covalent attachment of cell-adhesive, (Arg-Gly-Asp)-containing peptides to titanium surfaces. *Langmuir* **14**, 5507-5516 (1998).
118. Ferris, D. M., Moodie, G. D., Dimond, P. M., Gioranni, C. W. D., Ehrlich, M. G. & Valentini, R. F. RGD-coated titanium implants stimulate increased bone formation in vivo. *Biomaterials* **20**, 2323-2331 (1999).
119. Senyah, N., Hildebrand, G. & Liefeith, K. Comparison between RGD-peptide-modified titanium and borosilicate surfaces. *Analytical & Bioanalytical Chemsitry* **383**, 758-762 (2005).
120. Boateng, S. Y., Lateef, S. S., Mosley, W., Hartman, T. J., Hanley, L. & Russell, B. RGD and YIGSR synthetic peptides facilitate cellular adhesion identical to that of laminin and fibronectin but alter the physiology of neonatal cardiac myocytes. *American Journal of Physiology - Cell Physiology* **288**, 30-38 (2004).
121. Lateef, S. S., Boateng, S. Y., Hartman, T. J., Crot, C. A., Russell, B. & Hanley, L. GRGDSP peptide-bound silicone membranes withstand mechanical flexing in vitro and display enhanced fibroblast adhesion. *Biomaterials* **23**, 3159-3168 (2002).
122. Swan, E. E. L., Popat, K. C. & Desai, T. A. Peptide-immobilized nanoporous alumina membranes for enhanced osteoblast adhesion. *Biomaterials* **26**, 1969-1976 (2005).
123. Rezania, A. & Healy, K. E. Biomimetic Peptide Surfaces that Regulate Adhesion, Spreading, Cytoskeletal Organization and Mineralization of the Matrix Deposited by Osteoblast-like Cells. *Biotechnology Progress* **15**, 19-32 (1999).
124. Rezania, A., Johnson, R., Lefkow, A. R. & Healy, K. E. Bioactivation of Metal Oxide Surfaces. 1. Surface Characterization and Cell Response. *Langmuir* **15**, 6931-6939 (1999).
125. Rezania, A. & Healy, K. E. The effect of peptide surface density on mineralization of a matrix deposited by osteogenic cells. *Journal of Biomedical Materials Research* **52**, 595-600 (2000).
126. Dee, K. C., Rueger, D. C., Andersen, T. T. & Bizios, R. Conditions which promote mineralization at the bone-implant interface: a model *in vitro* study. *Biomaterials* **17**, 209-215 (1996).
127. Dee, K. C., Anderson, T. T. & Bizios, R. Osteoblast Population Migration Characteristics on Substrates Modified with Immobilized Adhesive Peptides. *Biomaterials* **20**, 221-227 (1999).

128. Olbrich, K. C., Anderson, T. T., Blumenstock, F. A. & Bizios, R. Surfaces modified with covalently-immobilized adhesive peptide affect fibroblast population motility. *Biomaterials* **17**, 759-764 (1996).
129. Massia, S. P. & Hubbell, J. A. An RGD Spacing of 440 nm is Sufficient for Integrin $\alpha_v\beta_3$-mediated Fibroblast Spreading and 140 nm for Focal Contact and Stress Fibre Formation. *Journal of Cell Biology* **114**, 1089-1100 (1991).
130. Massia, S. P. & Hubbell, J. A. Vascular Endothelial Cell Adhesion and Spreading Promoted by the Peptide REDV of the IIICS Region of Plasma Fibronectin is Mediated by Integrin $\alpha_4\beta_1$. *Journal of Biological Chemistry* **267**, 14019-14026 (1992).
131. Mann, B. K., Tsai, A. T., Scott-Burden, T. & West, J. L. Modification of Surfaces with Cell Adhesion Peptides Alters Extracellular Matrix Deposition. *Biomaterials* **20**, 2281-2286 (1999).
132. Porte-Durrieu, M. C., Labrugere, C., Villars, F., Lefebvre, F., Dutoya, S., Guette, A., Bordenave, L. & Baquey, C. Development of RGD peptides grafted onto silica surfaces: XPS characterization and human endothelial cell interactions. *Journal of Biomedical Materials Research* **46**, 368-375 (1999).
133. Lodish, Berk, Zipursky, Matsudaira, Baltimore & Darnell. (W H Freeman and Company, 2000).
134. Cooper, G. M. *The Cell: A Molecular Approach* (Sinauer, 2000).
135. Eckert, R., Jeney, S. & Horber, J. K. H. Understanding intercellular interactions and cell adhesion: lessons from studies on protein-metal interactions. *Cell Biology International* **21**, 707-713 (1997).
136. Faucheux, N., Schweiss, R., Lutzow, K., Werner, C. & Groth, T. Self-assembled monolayers with different terminating groups model substrates for cell adhesion studies. *Biomaterials* **25**, 2721-2730 (2004).
137. Xu, L.-C. & Siedlecki, C. A. Effects of surface wettability and contact time on protein adhesion to biomaterial surfaces. *Biomaterials* **28**, 3273-3283 (2007).
138. Andrade, J. D., Hlady, V. & Wei, A. P. Adsorption of complex proteins at interfaces. *Pure & Applied Chemistry* **64**, 1777-1781 (1992).
139. Fang, F. & Szleifer, I. Kinetics and thermodynamics of protein adsorption: a generalized molecular theoretical approach. *Biophysical Journal* **80**, 2568-2589 (2001).
140. Kasemo, B. & Gold, J. Implant surfaces and interface processes. *Adv. Dent. Res.* **13**, 8-20 (1999).
141. Sunami, H., Ito, E., Tanaka, M., Yamamoto, S. & Shimomura, M. Effect of honeycomb film on protein adsorption cell adhesion and proliferation. *Colloids and Surfaces A: Physiochem. Eng. Aspects* **284-285**, 548-551 (2006).
142. Chittur, K. K. FITR / ATR for protein adsorption to biomaterial surfaces. *Biomaterials* **19**, 357-369 (1998).
143. Zhang, Y., Chai, C., Jiang, X. S., Teoh, S. H. & Leong, K. W. Fibronectin immobilized by covalent conjugation or physical adsorption shows different bioactivity on aminated-PET. *Materials Science & Engineering C* **27**, 213-219 (2007).
144. Haynes, C. A. & Norde, W. Globular proteins at solid / liquid interfaces. *Colloids and Surfaces B: Biointerfaces* **2**, 517-566 (1994).
145. Welzel, P. B. Investigation of adsorption-induced structural changes of proteins at solid/liquid interfaces by differential scanning calorimetry. *Thermochimica Acta* **382**, 175-188 (2002).

146. Yongli, C., Xiufang, Z., Yandao, G., Nanming, Z., Tingying, Z. & Xinqi, S. Conformational Changes of Fibrinogen Adsorption onto Hydroxyapatite and Titanium Oxide Nanoparticles. *Journal of Colloid and Interface Science* **214**, 38-45 (1999).
147. Ding, Y., Shu, Y. & Guo, R. The effect of sodium dodecyl sulface on the conformation of bovine serum albumin. *Colloids and Surfaces A: Physiochem. Eng. Aspects* **298**, 163-169 (2007).
148. Long, S. F., Clarke, S., Davies, M. C., Lewis, A. L., Hanlon, G. W. & Lloyd. Controlled biological response on blends of a phosphorylcholine-based copolymer with poly(butyl methacrylate). *Biomaterials* **24**, 4115-4121 (2003).
149. Lee, J. H., Khang, G., Lee, J. W. & Lee, H. B. Interaction of different types of cells on polymer surfaces with wettability gradient. *Journal of Colloid and Interface Science* **205**, 323-330 (1998).
150. Lee, M. H., Ducheyne, P., Lynch, I., Boettiger, D. & Composto, R. J. Effect of biomaterial surface properties on fibronectin–$\alpha_5\beta_1$ integrin interaction and cellular attachment. *Biomaterials* **27**, 1907-1916 (2006).
151. Allen, L. T., Tosetto, M., Miller, I. S., O'Connor, D. P., Penney, S. C., Lynch, I., Keenan, A. K., Pennington, S. R., Dawson, K. A. & Gallagher, W. M. Surface-induced changes in protein adsorption and implications for cellular phenotypic responses to surface interaction. *Biomaterials* **27**, 3096-3108 (2006).
152. Fitchmin, M. I., Falk-Marzillier, J., Marshall, E., Cruz, G., Jones, J. C. R. & Quaranta, V. Mode of adsorption and orientation of an extracellular matrix protein affect its cell-adhesion promoting activity. *Analytical Biochemistry* **265**, 1-7 (1998).
153. Keselowsky, B. G. Surface chemistry modulates focal adhesion composition and signaling through changes in integrin binding. *Biomaterials* **25**, 5947-5954 (2004).
154. Lan, M. A., Gersbach, C. A., Michael, K. E., Keselowsky, B. G. & Garcia, A. J. Myoblast proliferation and differentiation on fibronectin-coated self assembled monolayers presenting different surface chemistries. *Biomaterials* **26**, 4523-4531 (2005).
155. Lord, M. S., Cousins, B. G., Doherty, P. J., Whitelock, J. M., Simmons, A., Williams, R. L. & Milthorpe, B. K. The effect of silica nanoparticulate coatings on serum protein adsorption and cellular response. *Biomaterials* **27**, 4856-4862 (2006).
156. Reno, F., Lombardi, F. & Cannas, M. Surface-adsorbed α_1-microglobulin modulation of human fibroblasts spreading and matrix metalloproteinases release. *Biomaterials* **25**, 3439-3443 (2004).
157. Ruoslahti, E. & Piersbacher, M. D. Arg-Gly-Asp: A versatile cell recognition signal. *Cell* **44**, 517-518 (1986).
158. Pierschbacher, M. D. & Ruoslahti, E. Cell Attachment Activity of Fibronectin can be Duplicated by Small Fragments of the Molecule. *Nature* **309**, 30-33 (1984).
159. Hersel, U., Dahmen, C. & Kessler, H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. *Biomaterials* **24**, 4385-4415 (2003).
160. Ruoslahti, E. RGD and Other Recognition Sequences for Integrins. *Annu. Rev. Cell. Dev. Biol.* **12**, 697-715 (1996).
161. Hayman, E. G., Piersbacher, M. D. & Ruoslahti, E. Detachment of Cells from Culture Substrate by Soluble Fibronectin Peptides. *Journal of Cell Biology* **100**, 1948-1954 (1985).

162. Elloumi, I., Kobayashi, R., Funabashi, H., Mie, M. & Kobatake, E. Construction of epidermal growth factor fusion protein with cell adhesive activity. *Biomaterials* **27**, 3451-3458 (2006).
163. Underwood, P. A., Steele, J. G. & Dalton, B. A. Effects of polystyrene surface chemistry on the biological activity of solid phase fibronectin and vitronectin, analysed with monoclonal antibodies. *Journal of Cell Science* **104**, 793-803 (1993).
164. Buckley, C. D., Pilling, D., Henriquez, N. V., Parsonage, G., Threlfall, K., Scheel-Toellner, D., Simmons, D. L., Akbar, A. N., Lord, J. M. & Salmon, M. RGD peptides induce apoptosis by direct caspase-3 activation. *Nature* **197**, 534-539 (1999).
165. Sawyer, A. A., Weeks, D. M., Kelpke, S. S., McCracken, M. S. & Bellis, S. L. The effect of the addition of a polyglutamate motif to RGD on peptide tethering to hydroxyapatite and the promotion of mesenchymal stem cell adhesion. *Biomaterials* **26**, 7046-7056 (2005).
166. Kurihara, H., Shinkai, M. & Nagamune, T. Microbial expression of proteins containing long repetitive Arg-Gly-Asp cell adhesive motifs created by overlap elongation PCR. *Biochemical and Biophysical Research Communications* **321**, 988 (2004).
167. Fujisawa, R., Mizuno, M., Nodasaka, Y. & Kuboki, Y. Attachment of Osteoblastic Cells to Hydroxyapatite Crystals by a Synthetic Peptide (Glu_7-Pro-Arg-Gly-Asp-Thr) Containing Two Functional Sequences of Bone Sialoprotein. *Matrix Biology* **16**, 21-28 (1997).
168. Ernsting, M. J., Bonin, G. C., Yang, M., Labow, R. S. & Santerre, J. P. Generation of cell adhesive substrates using peptide fluoralkyl surface modifiers. *Biomaterials* **26**, 6536-6546 (2005).
169. Pakalns, T., Haverstick, K. L., Fields, G. B., McCarthy, J. B., Mooradian, D. L. & Tirrell, M. Cellular Recognition of Synthetic Peptide Amphiphiles in Self-Assembled Monolayer Films. *Biomaterials* **20**, 2265-2279 (1999).
170. Mardilovich, A., Craig, J. A., McCammon, M. Q., Garg, A. & Kokkoli, E. Design of a Novel Fibronectin-Mimetic Peptide-Amphiphile for Functionalized Biomaterials. *Langmuir* **22**, 3259-3264 (2006).
171. Houseman, B. T. & Mrksich, M. The microenvironment of immobilized Arg-Gly-Asp peptides is an important determinant of cell adhesion. *Biomaterials* **22**, 943-955 (2001).
172. Biesalski, M. A., Knaebel, A., Tu, R. & Tirrell, M. Cell adhesion on a polymerized peptide-amphiphile monolayer. *Biomaterials* **27**, 1259-1269 (2006).
173. Ikada, Y. Surface modification of polymers for biomedical applications. *Biomaterials* **15**, 725-736 (1994).
174. Aslam, M. & Dent, A. *Bioconjugation: Protein Coupling for the Biomedical Sciences* (Macmillan Reference Ltd, 1998).
175. Reinhart-King, C. A., Dembo, M. & Hammer, D. A. Endothelial Cell Traction Forces on RGD-Derivatized Polyacrylamide Substrate. *Langmuir* **19**, 1573-1579 (2003).
176. Lee, M. H., Brass, D. A., Morris, R., Composto, R. J. & Ducheyne, P. The effect of non-specific interactions on cellular adhesion using model surfaces. *Biomaterials* **26**, 1721-1730 (2005).
177. Staros, J. V., Wright, R. W. & Swingle, D. M. Enhancement by N-hydroxysulfosuccinimde of water-soluble carbodiimide-mediated coupling reactions. *Analytical Biochemistry* **156**, 220-222 (1986).

178. Park, K.-H., Na, K. & Lee, K. C. Immobilization of Arg-Gly-Asp (RGD) Sequence in Sugar Containing Copolymer for Culturing of Pheochromocytoma (PC12) Cells. *Journal of Bioscience and Bioengineering* **97**, 207-211 (2004).
179. Yamaoka, T., Hotta, Y., Kobayashi, K. & Kimura, Y. Synthesis and Properties of Malic Acid Containing Functional Polymers. *International Journal of Biological Macromolecules* **25**, 265-271 (1999).
180. Schnaar, R. L., Langer, B. G. & Brandley, B. K. Reversible Covalent Immobilisation of Ligands and Proteins on Polyacrylamide Gels. *Analytical Biochemistry* **151**, 268-281 (1985).
181. Carlisle, E. S., Muthumarthanda, M. S., Mariappan, R., Nelson, K. D., Thomes, B. E., Timmons, R. B., Constantinescu, A., Eberhart, R. C. & Bankey, P. E. Enhancing Hepatocyte Adhesion by Pulsed Plasma Depostion and Polyethylene Glycol Coupling. *Tissue Engineering* **6**, 45-52 (2000).
182. Tong, Y. W. & Shoichet, M. S. Peptide surface modification of poly(tetrafluoroethylene-co-hexafluoropropylene) enhances its interaction with central nervous system neurons. *Journal of Biomedical Materials Research* **42**, 85-95 (1998).
183. Massia, S. P. & Hubbell, J. A. Covalent surface immobilisation of Arg-Gly-Asp and Tyr-Ile-Gly-Ser-Arg-containing peptides to obtain well-defined cell-adhesive substrates. *Analytical Biochemistry* **187**, 292-301 (1990).
184. Ji, J., Zhu, H. & Shen, J. Surface tailoring of poly(DL-lactic acid) by ligand-tethered amphiphilic polymer for promoting chondrocyte attachment and growth. *Biomaterials* **25**, 1859-1867 (2004).
185. Kato, M. & Mrksich, M. Using Model Substrates To Study the Dependence of Focal Adhesion Formation on the Affinity of Integrin-Ligand Complexes. *Biochemistry* **43**, 2699-2707 (2004).
186. Glass, J. R., Dickerson, K. T., Stecker, K. & Polarek, J. W. Characterization of a halouronic acid-Arg-Gly-Asp peptide cell attachment matrix. *Biomaterials* **17**, 1101-1108 (1996).
187. Massia, S. P. & Stark, J. Immobilized RGD Peptides on Surface-Grafted Dextran promote Biospecific Cell Attachment. *Journal of Biomedical Materials Research* **56**, 390-399 (2001).
188. Walluscheck, K. P., Steinhoff, G., Kelm, S. & Haverich, A. Improved Endothelial Cell Attachment on ePTFE Vascular Grafts Pretreated with Synthetic RGD-containing Peptides. *Eur. J. Vasc. Endovasc. Surg.* **12**, 321-330 (1996).
189. Holland, J., Hersh, L., Bryhan, M., Onyiriuka, E. & Ziegler, L. Culture of Human Vascular Endothelial Cells on an RGD-containing Synthetic Peptide Attached to a Starch-Coated Polystyrene Surface: Comparison with Fibronectin-Coated Tissue Grade Polystyrene. *Biomaterials* **17**, 2147-2156 (1996).
190. Ivanov, B., Grzesik, W. & Robey, F. A. Synthesis and Use of a New Bromoacetyl-Derivatized Heterotrifunctional Amino Acid for Conjugation of Cyclic RGD-Containing Peptides Derived from Human Bone Sialoprotein. *Bioconjugate Chemistry* **6**, 269-277 (1995).
191. Healy, K. E., Rezania, A. & Stile, R. A. Designing Biomaterials to Direct Biological Responses. *ANNALS NEW YORK ACADEMY OF SCIENCES*, 24-35 (1999).
192. Sun, H., Wirsen, A. & Albertsson, A.-C. Electron Beam-Induced Graft Polymerization of Acrylic Acid and Immobilization of Arginine-Glycine-Aspartic Acid-Containing Peptide onto Nanopatterned Polycaprolactone. *Biomacromolecules* **5**, 2275-2280 (2004).

193. Wirsen, A., Sun, H., Emilsson, L. & Albertsson, A.-C. Solvent Free Vapor Phase Photografting of Maleic Anhydride onto Poly(ethylene terephthalate) and Surface Coupling of Fluorinated Probes, PEG, and an RGD-Peptide. *Biomacromolecules* **6**, 2281-2289 (2005).
194. Hermanson, G. T. *Bionconjugate Techniques* (Academic Press, 1996).
195. Herbert, C. B., McLernon, T. L., Hypolite, C. L., Adams, D. N., Pikus, L., Huang, C.-C., Fields, G. B., Letourneau, P. C., Distefano, M. D. & Hu, W.-S. Micropatterning gradients and controlling surface densities of photoactivatable biomolecules on self-assembled monolayers of oligo(ethylene glycol) alkanethiolates. *Chemistry & Biology* **4**, 731-737 (1997).
196. Singer, I. I., Scott, S., Kawka, D. W., Kazazis, D. M., Gailit, J. & Ruoslahti, E. Cell Surface Distribution of Fibronection and Vitronectin Receptors Depends on Substrate Composition and Extracellular Matrix Accumulation. *Journal of Cell Biology* **106**, 2171-2182 (1988).
197. Streeter, H. B. & Rees, D. A. Fibroblast Adhesion to RGDS Shows Novel Features Compared with Fibronectin. *Journal of Cell Biology* **105**, 507-515 (1987).
198. Zheng, H., Berg, M. C., Rubner, M. F. & Hammond, P. T. Controlling Cell Attachment Selectively onto Biological Polymer-Colloid Templates Using Polymer-on-Polymer Stamping. *Langmuir* **20** (2004).
199. Berg, M. C., Yang, S. Y., Hammond, P. T. & Rubner, M. F. Controlling Mammalian Cell Interactions on Patterned Polyelectrolyte Multilayer Surfaces. *Langmuir* **20**, 1362-1368 (2004).
200. Balasundaram, G., Sato, M. & Webster, T. J. Using hydroxyapatite nanoparticles and decreased crystallinity to promote osteoblast adhesion similar to functionalizing with RGD. *Biomaterials* **27**, 2798-2805 (2006).
201. Porte-Durrieu, M. C., Guillemot, F., Pallu, S., Labrugere, C., Brouillard, B., Bareille, R., Amedee, J., Barthe, N., Dard, M. & Baquey, C. Cyclo-(DfKRG) peptide grafting onto Ti–6Al–4V: physical characterization andinterest towards human osteoprogenitor cells adhesion. *Biomaterials* **25**, 4837-4846 (2004).
202. Cutler, S. M. & Garcia, A. J. Engineering Cell Adhesive Surfaces that Direct Integrin a5b1 Binding using a Recombinant Fragment of Fibronectin. *Biomaterials* **24**, 1799-1720 (2003).
203. Pless, P. D., Lee, Y. C., Roseman, S. & Schnaar, R. L. Specific Cell Adhesion to Immobilized Glycoproteins Demonstrated Using New Reagents for Protein and Glycoprotein Immobilization. *Journal of Biological Chemistry* **258**, 2340-2349 (1983).
204. Ito, A., Mase, A., Takizawa, Y., Shinkai, M., Honda, H., Hata, K.-I., Ueda, M. & Kobayashi, K. Transglutaminase-Mediated Gelatin Matrices Incorporating Cell Adhesion Factors as a Biomaterials for Tissue Engineering. *Journal of Bioscience and Bioengineering* **95**, 196-199 (2003).
205. Meinhart, J. G., Schense, J. C., Schima, H., Gorlitzer, M., Hubbell, J. A., Deutsch, M. & Zilla, P. Enhanced Endothelial Cell Retention on Shear-Stressed Synthetic Vascular Grafts Precoated with RGD-Cross-Linked Fibrin. *Tissue Engineering* **11**, 887-894 (2005).
206. Tosatti, S., De Paul, S. M., Askendal, A., VandeVondele, S., Hubbell, J. A., Tengvall, P. & Textor, M. Peptide functionalized poly(l-lysine)-g-poly(ethylene glycol) on titanium: resistance to protein adsorption in full heparinized human blood plasma. *Biomaterials* **24**, 4949-4958 (2003).
207. Singer, I. I., Kawka, D. W., Scott, S., M umford, R. A. & Lark, M. W. The Fibronectin Cell Attachment Sequence Arg-Gly-Asp-Ser Promotes Focal

Contact Formation during Early Fibroblast Attachment and Spreading. *Journal of Cell Biology* **104**, 573-584 (1987).
208. Maheshwari, G., Brown, G., Lauffenburger, D. A., Wells, A. & Griffiths, L. G. Cell Adhesion and Motility Depend on Nanoscale RGD Clustering. *Journal of Cell Science* **113**, 1677-1686 (2000).
209. Fussell, G. W. & Cooper, S. L. Synthesis and characterization of acrylic terpolymers with RGD peptides for biomedical applications. *Biomaterials* **25**, 2971-2978 (2004).
210. Sawyer, A. A., Hennessy, K. M. & Bellis, S. L. Regulation of mesenchymal stem cell attachment and spreading on hydroxyapatite by RGD peptides and adsorbed serum proteins. *Biomaterials* **26**, 1467-1475 (2005).
211. Parniak, M. A., Lange, G. & Viswanatha, T. Quantitiative determination of monosubstituted guanidines: a comparative study of different procedures. *Journal of Biochem. Biophys. Methods* **7**, 267-76 (1983).
212. Park, K.-H. & Bae, Y. H. Phenotype of Hepatocyte Spheroids in Arg-Gly-Asp (RGD) Containing a Thermo-Reversible Extracellular Matrix. *Biosci. Biotechnol. Biochem.* **66**, 1473-1478 (2002).
213. Park, K.-H. Arg-Gly-Asp (RGD) Sequence Conjugated in a Synthetic Copolymer Bearing a Sugar Moiety for Improved Culture of Parenchymal Cells (Hepatocytes). *Biotechnology Letters* **24**, 1401-1406 (2002).
214. Malek, A. M., Lee, I. W., Alper, S. L. & Izumo, S. Regulation of Endothelin-1 Gene Expression by Cell Shape and the Microfilament Network in Vascular Endothelium. *American Journal of Physiology* **273**, 1764-1774 (1997).
215. Plow, E. F., Haas, T. A., Zhang, L., Loftus, J. & Smith, J. W. Ligand Binding to Integrins. *Journal of Biological Chemistry* **275**, 21785-21788 (2000).
216. Hynes, R. O. Integrins: Versatility, Modulation, and Signalling in Cell Adhesion. *Cell* **69**, 11-25 (1992).
217. Sinha, R. K. & Tuan, R. S. Regulation of Human Osteoblast Integrin Expression by Orthopedic Implant Materials. *Bone* **18**, 451-457 (1996).
218. van der Flier, A. & Sonnenberg, A. Function and interactions of integrins. *Cell Tissue Research* **305**, 285-298 (2001).
219. Clyman, R. I., Mauray, F. & Kramer, R. H. β1 and β3 Integrins Have Different Roles in the Adhesion and Migration of Vascular Smooth Muscle Cells on Extracellular Matrix. *Experimental Cell Research* **200**, 272-284 (1992).
220. Petit, V. & Thiery, J.-P. Focal adhesions: structure and dynamics. *2000* **92**, 477-494 (2000).
221. Cook, A. D., Hrkach, J. S., Gao, N. N., Johnson, I. M. & Pajvani, U. B. Characterisation and Development of RGD-peptide Modified Poly(Lactic acid-co-Lysine) as an Interactive Resorbable Biomaterial. *Journal of Biomedical Materials Research* **35**, 513-523 (1997).
222. Grzesiak, J. J., Piersbacher, M. D., Amodeo, M. F., Malaney, T. I. & Glass, J. R. Enhancement of cell interactions with collagen/glycosaminoglycan matrices by RGD derivatisation. *Biomaterials* **19**, 1625-1632 (1997).
223. Jeschke, B., Meyer, J., Jonczyk, A., Kessler, H., Adamietz, P., Meenen, N. M., Kantlehner, M., Goepfert, C. & Nies, B. RGD-peptides for tissue engineering of articular cartilage. *Biomaterials* **23**, 3455-3463 (2002).
224. Tiwari, A., Kidane, A., Salacinski, H., Punshon, G., Hamilton, G. & Seifalian, A. M. Improving Endothelial Cell Retention for Single Stage Seeding of Prosthetic Grafts: Use of Polymer Sequences of Arginine-Glycine-Aspartate. *Eur. J. Vasc. Endovasc. Surg.* **25**, 325-329 (2003).

225. Miyata, T., Conte, M. S., Trudell, L. A., Mason, D., Whittemore, A. D. & Birinyi, L. K. Delayed Exposure to Pulsatile Shear Stress Improves Retention of Human Saphenous Vein Endothelial Cells on Seeded ePTFE Grafts. *Journal of Surgical Research* **50**, 485-493 (1991).
226. Lee, Y.-J., Park, S.-J., Lee, W.-K., Ko, J. S. & Kim, H.-M. MG63 osteoblastic cell adhesion to the hydrophobic surface precoated with recombinant osteopontin fragments. *Biomaterials* **24**, 1059-1066 (2003).
227. Deng, C., Tian, H., Zhang, P., Sun, J., Chen, X. & Jing, X. Synthesis and Characteriztion of RGD Peptide Grafted Poly(ethylene glycol)-b-Poly(L-lactide)-b-Poly(L-glutamic acid) Triblock Copolymer. *Biomacromolecules* **7**, 590-596 (2006).
228. Pallu, S., Bareille, R., Dard, M., Kessler, H., Jonczyk, A., Vernizeau, M. & Amedee-Vilamitjana, J. A cyclo peptide activates signaling events and promotes growth and the production of the bone matrix. *Peptides* **24**, 1349-1357 (2003).
229. Lazar, A., Mann, H. J., Remmel, R. P., Shatford, R. A., Cerra, F. B. & Hu, W. S. Formation of porcine hepatocyte spheroids for use in a bioartificial liver. *Cell Transplant.* **4**, 259-268 (1995).
230. Park, K.-H. Improved Long-Term Culture of Hepatocytes in a Hydrogel Containing Arg-Gly-Asp (RGD). (2002).
231. Woessner, J. F. The determination of hydroxyproline in tissue, protein samples contraining small proportion of this imino acid. *Arch. Biochem. Biophys.* **93**, 440-447 (1961).
232. Janssen, M. I., van Leeuwen, M. B. M., Scholtmeijer, K., van Kooten, T. G., Dijkhuizen, L. & Wosten, H. A. B. Coating with genetic engineered hydrophobin promotes growth of fibroblasts on a hydrophobic solid. *Biomaterials* **23**, 4847-4854 (2002).
233. Rocková-Hlavácková, K., Svorcik, V., Bacakova, L., Dvorankova, B., Heitz, J. & Hnatowicz. Bio-compatibility of ion beam-modified and RGD-grafted polyethylene. *Nuclear Instruments and Methods in Physics Research B* **225**, 275-282 (2004).
234. Holly, S. P., Larson, M. K. & Parise, L. V. Multiple Roles of Integrins in Cell Motility. *Experimental Cell Research* **261**, 69-64 (2000).
235. Katz, B.-Z., Zamir, E., Bershadsky, A., Kam, Z., Yamada, K. M. & Geiger, B. Physical State of the Extracellular Matrix Regulates the Structure and Molecular Composition of Cell-Matrix Adhesions. *Molecular Biology of the Cell* **11**, 1047-1060 (2000).
236. Behravesh, E., Sikavitsas, V. I. & Mikos, A. G. Quantification of ligand surface concentration of bulk-modified biomimetic hydrogel. *Biomaterials* **24**, 4365-4374 (2003).
237. Xiong, J.-P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimiak, A., Goodman, S. L. & Arnaout, M. A. Crystal Structure of the Extracellular Segment of Integrin αvβ3. *Science* **294**, 339-345 (2001).
238. Xiong, J.-P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L. & Arnaout, M. A. Crystal Structure of the Extracellular Segment of Integrin αvβ3 in Complex with an Arg-Gly-Asp Ligand. *Science* **296**, 151-155 (2002).
239. Hautanen, A., Gailit, J., Mann, D. M. & Ruoslahti, E. Effect of Modifications of the RGD Sequence and Its Context on Recognition by the Fibronectin Receptor. *Journal of Biological Chemistry* **264**, 1437-1442 (1989).
240. Cheng, S., Craig, W. S., Mullen, D., Tschopp, J. F., Dixon, D. & Piersbacher, M. D. Design and Synthesis of Novel Cyclic RGD-Containing

Peptides as Highly Potent and Selective Integrin αIIbβ3 Antagonists. *Journal of Medicinal Chemistry* **37**, 1-8 (1994).
241. Pierschbacher, M. D. & Ruoslahti, E. Influence of Stereochemistry of the Sequence Arg-Gly-Asp-Xaa on Binding Specificity in Cell Adhesion. *Journal of Biological Chemistry* **262**, 17294-17298 (1987).
242. Lanza, P., Felding-Habermann, B., Ruggeri, Z. M., Zanetti, M. & Billetta, R. Selective Interaction of a Conformationally-constrained Arg-Gly-Asp (RGD) Motif with the Integrin Receptor avb3 Expressed on Human Tumor Cells. *Blood Cells, Molecular and Diseases* **23**, 230-241 (1997).
243. Aumailley, M., Gurrath, M., Muller, G., Calvete, J., Timpl, R. & Kessler, H. Arg-Gly-Asp constrained within cyclic pentapeptides. *FEBS* **291**, 50-54 (1991).
244. Marchi-Artzner, V., Lorz, B., Hellerer, U., Kantlehner, M., Kessler, H. & Sackmann, E. Selective Adhesion of Endothelial Cells to Artificial Membranes with a Synthetic RGD-Lipopeptide. *Chem. Eur. J.* **7**, 1095-1101 (2001).
245. Pfaff, M., Tangemann, K., Muller, B., Gurrath, M., Muller, G., Kessler, H., Timpl, R. & Engel, J. Selective Recognition of Cyclic RGD Peptides of NMR Defined Conformation by αIIBβ3, αVβ3 and α5β1 Integrins. *Journal of Biological Chemistry* **269**, 20233-20238 (1994).
246. Gurrath, M., Muller, G., Kessler, H., Aumailley, M. & Timpl, R. Conformation / activity studies of rationally designed potent anti-adhesive RGD peptides. *European Journal of Biochemistry* **210**, 911-912 (1992).
247. Irvine, D. J., Hue, K.-A., Mayes, A. M. & Griffith, L. Simulations of Cell-Surface Integrin Binding to Nanoscale-Clustered Adhesion Ligands. *Biophysical Journal* **82**, 120-132 (2002).
248. Roberts, C., Chen, C. S., Mrksich, M., Martichonok, V., Ingber, D. E. & Whitesides, G. M. Using Mixed Self-Assembled Monolayers Presenting RGD and $(EG)_3OH$ Groups to Characterize Long-Term Attachment of Bovine Capillary Endothelial Cells to Surfaces. *Journal of the American Chemical Society* **120**, 6548-6555 (1998).
249. Sagnella, S. M., Kilgman, F., Anderson, E. H., King, J. E., Murugesan, G., Marchant, M. E. & Kottke-Marchant, K. Human microvascular endothelial cell growth and migration on biomimetic surfactant polymers. *Biomaterials* **25**, 1249-1259 (2004).
250. Schaffner, P., Meyer, J., Dard, M., Wenz, R., Nies, B., Verrier, S., Kessler, K. & Kantlehner, M. Induced Tissue Integration of Bone Implants by Coating with Bone Selective RGD-peptides In Vitro and In Vivo Studies. *Journal of Materials Science: Materials in Medicine* **10**, 837-839 (1999).
251. Sagnella, S. M., Anderson, E. H., Sanabria, N., Marchant, R. E. & Kottke-Marchant, K. Human Endothelial Cell Interaction with Biomimetic Surfactant Polymers Containing Peptide Ligands from the Heparin Binding Domain of Fibronectin. *Tissue Engineering* **11**, 226-236 (2005).
252. Small, J. V., Kaverina, I., Krylyshkina, O. & Rottner, K. Cytoskeleton cross-talk during cell motility. *FEBS Letters* **452**, 96-99 (1999).
253. Palacek, S. P., Loftus, J., Ginsberg, M. H., Lauffenburger, D. A. & Horwitz, A. F. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* **385**, 537-540 (1997).
254. Smilenov, L. B., Mikhailov, A., Pelham, R. J., Marcantonio, E. E. & Gundersen, G. G. Focal Adhesion Motility Revealed in Stationary Fibroblasts. *Science* **286**, 1172-1174 (1999).

255. Simon, K. O., Nutt, E. M., Abraham, D. G., Rodan, G. A. & Duong, L. T. The αvβ3 Integrin Regulates α5β1-mediated Cell Migration toward Fibronectin. *Journal of Biological Chemistry* **272**, 29380-29389 (1997).
256. Connelly, J. T., Garcia, A. J. & Levenston, M. E. Inhibition of in vitro chondrogenesis in RGD-modified three-dimensional alginate gels. *Biomaterials* **28**, 1071-1083 (2007).
257. Schliephake, H., Scharnweber, D., Dard, M., Roβler, S., Sewing, A., Meyer, J. & Hoogestraat, D. Effect of RGD peptide coating of titanium implants on periimplant bone formation in the alveolar crest. *Clinical Oral Implant Research* **13**, 312-319 (2002).
258. Saneinejad, S. & Schoichet, M. S. Patterned glass surfacs direct cell adhesion and process outgrowth of primary neurons of the central nervous system. *Journal of Biomedical Materials Research* **42**, 13-19 (1998).
259. Levesque, S. G. & Shoichet, M. S. Synthesis of cell-adhesive dextran hydrogels and macroporous scaffolds. *Biomaterials* **27**, 5277-5285 (2006).
260. Jasti, S. (Aldrich Chemical Co., Inc).
261. Sun, Y., Collett, J., Fullwood, N., MacNeil, S. & Rimmer, S. Culture of dermal fibroblasts and protein adsorption on block conetworks of poly(butyl methacrylate-block-(2,3 propandiol-1-methacrylate-stat-ethandiol dimethacrylate)). *Biomaterials* **28**, 661-670 (2007).
262. Zhao, Z., Koeplinger, K. A., Peterson, T., Conradi, R. A., Burton, P. S., Suarato, A., Heinrikson, R. L. & Tomasselli, A. G. Mechanism, Structure-Activity Studies and Potential Applications of Glutathione-S-Transferase-Catalyzed Cleavage of Sulfonamides. *Drug Metabolism and Disposition* **27**, 992-998 (1999).
263. Jeyaraj, D. A. & Waldmann, H. Synthesis of nucleopeptides by an enzyme labile urethane protecting group. *Tetrahedron letters* **42**, 835-837 (2001).
264. Kappes, T. & Waldmann, H. The tetrabenzylglucosyloxycarbonyl(BGloc)-group-Acarbohydrate-derived enzyme-labile urethane protecting group. *Carbohydrate research* **305**, 341 (1998).
265. Muller, G. H. & Waldmann, H. The Phenyl Hydrazide as an Enzyme-labile Protecting Group - Oxidative Cleavage with Mushroom Tyrosinase. *Tetrahedron Letters* **40**, 3549-3552 (1999).
266. Chan, W. C. & White, P. D. *Fmoc Solid Phase Peptide Synthesis* (ed. Hames, B. D.) (Oxford University Press, 2000).
267. Hoeg-Jensen, T., Jakobsen, M. H., Olsen, C. E. & Holm, A. Formation of Peptide Thioamides by Use of Fmoc Monothioacids and PyBOP. *Tetrahedron Letters* **32**, 7617-7620 (1991).
268. Ramage, R., Green, J. & Blake, A. J. An Acid Labile Arginine Derivative for Peptide Synthesis: NG-2,2,5,7,8-Pentamethylchroman-6-sulphonyl-L-arginine. *Tetrahedron* **47**, 6353-6370 (1991).
269. Carpino, L. A. & Han, G. Y. The 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. *Journal of Organic Chemistry* **57**, 3404-3409 (1972).
270. Rink, H. Solid-Phase Synthesis of Protected Peptide Fragments Using a Trialkoxy-diphenyl-methylester Resin. *Tetrahedron Letters* **28**, 3787-3790 (1987).
271. Blankemeyer-Menge, B., Nimtz, M. & Frank, R. An Efficient Method for Anchoring Fmoc-Amino Acids to Hydroxyl-Functionalised Solid Supports. *Tetrahedron Letter* **31**, 1701-1704 (1990).
272. Coste, J., Le-Nguyen, D. & Castro, B. PyBOP:A New Peptide Coupling Reagent Devoid of Toxic By-product. *Tetrahedron Letters* **31**, 205-208 (1990).

273. Blanchfield, J., Dutton, J., Hogg, R., Craik, D., Adams, D., Lewis, R., Alewood, P. & Toth, I. The synthesis and structure of an n-terminal dodecanoic acid conjugate of α-conotoxin MII. *Letters in Peptide Science* **8**, 235-239 (2002).
274. Leinweber, F. J. & Monty, K. J. Sulfite determination: fuchsin method. *Methods in Enzymology* **143**, 15-17 (1987).
275. Grant, W. M. Colorimetric determination of sulphur dioxide. *Analytical Chemistry* **19**, 345-346 (1947).
276. Higham, M. C., Dawson, R., Szabo, M., Short, R., Haddow, D. B. & MacNeil, S. Development of a stable chemically defined surface for the culture of human keratinocytes under serum-free conditions for clinical use. *Tissue Engineering* **9**, 919-31 (2003).