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Transglutaminases as bonding agents for use in cartilage repair

Matthew Paul Evans

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Abstract

A consistent failure of cartilage repair technologies has been the secure anchoring of repair materials into defect sites. Until this is overcome, cartilage repair will continue to be compromised. The protein cross-linking, calcium-dependent enzyme tissue transglutaminase (tTG) has been proposed as an adhesive for binding cartilage-cartilage interfaces. Work presented in this thesis aimed to investigate the potential of tTG to act as a bonding agent for use in cartilage repair.

An *in vitro* test system was developed to measure tTG-catalysed bonding at cartilage-cartilage interfaces in response to tensile stress. tTG bond strength was found to be dependent on enzyme concentration, increasing incubation time and was consistent over a humidity range of 20-100%. Methods of pre-treating the tissue surfaces to improve bonding were investigated and enzymatic digestion of cartilage glycosaminoglycans (GAG's) proved most successful. To determine why this was the case, cartilage surfaces were analysed using various microscopy techniques. AFM analysis revealed GAG-digestion to be reducing the microscopic topography of the tissue surfaces, possibly allowing for more points of direct contact when opposed at an interface. Fluorescence microscopy showed that the digestion also exposes more potential TG-substrate, peptide-bound γ-glutamyl residues on the tissue surfaces. These may contribute to the increased bond strength of tTG at GAG-digested interfaces by allowing for a greater degree of cross-linking between the tissue surfaces. Biomaterials are increasingly important in cartilage repair and the present study demonstrates that tTG has the capacity to bond synthetic polymer biomaterials to GAG-digested cartilage surfaces. The materials were optimised for tTG-catalysed bonding by the inclusion of TG-substrate residues/proteins, indicating that cartilage repair materials may now be designed and optimised for maximum TG-catalysed retention at implant sites. A method was also developed for measuring chondrocyte viability at experimental wound edges in cartilage explants cultured *in vitro*. The loss of cell viability observed at the wound edges was decreased when the edge was cultured in contact with surrounding tissue, and increased by GAG-digestion of the tissue. It was unaffected by tTG-catalysed cross-linking of the wound edge interface, suggesting that tTG application to cartilage interfaces *in vivo* will not have a detrimental effect on cell viability at the interface surfaces.

Contents

List of abbreviations

AFM	Atomic Force Microscope
BMP	Bone Morphogenetic Protein
Ca^{2+}	Free calcium ion
CABC	Chondroitinase ABC
CAC	Chondroitinase AC
CaCl	Calcium chloride
CLSM	Confocal Laser Scanning Microscope
cm	Centimetre
CPPD	Crystal pyrophosphate dihydrate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECM	Extra-cellular matrix
EDTA	Ethylene Diamine Tetra Acetic Acid
ESEM	Environmental Scanning Electron Microscope
FC	Fluoroscein Cadaverine
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FN	Fibronectin
FXIII	Factor XIII
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate
Hep	Heparinase
Hya	Hyaluronidase
IGF	Insulin-like growth factor
IL	Interleukin
Kda	Kilodaltons
KG	Kilogram
KJ	Kilojoule
LTBP-1	Latent TGF-β1 Binding Protein-1

M	Molar
mg	Milligram
ml	Millilitres
mM	Millimolar
MPa	Mega pascalls
Mr	Molecular weight
mTG	Microbial Transglutaminase
µl	Microlitres
µM	Micromolar
N	Newton
NaCl	Sodium chloride
nm	Nanometre
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet Derived Growth Factor
pH	Negative log of hydrogen ion concentration
PGA	Polyglycolic Acid
PLGA	Poly-dl-lactic-co-gycolic Acid
PLLA	Poly-l-lactic Acid
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscope
TAB	Tris Activating Buffer
TBS	Tris buffered saline
TC	Texas-Red Cadaverine
TEM	Transmission Electron Microscope
TEMED	N,N,N,N'-teramethylene Diamine
TG	Transglutaminase
TGF-β1	Transforming growth factor β1
Tris	Tris(Hydroxymethyl)-aminoethane
tTG	Tissue Transglutaminase
U	Units
UV	Ultraviolet

V/V	Volume/Volume
W/V	Weight/Volume

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Chapter I : Introduction

Chapter I : Introduction

Wound closure during surgery is done mainly by mechanical fastening techniques such as suturing or stapling. However, over a range of surgical specialities these techniques are inadequate or unsuitable. Suturing requires skilled surgeons, is time-consuming and requires the post-operative removal of non-absorbable materials (as do clips and staples). Many tissues are also too delicate for mechanical devices to be placed therein and their location makes access difficult. An alternative option to these mechanical devices is the use of tissue adhesives.

I-1 Requirements of a tissue adhesive

The general properties of a tissue adhesive should be such that it is safe and effective, in terms of internal bonding strength, surface adherence, enhancing wound healing and tissue regeneration, being quick to use, easily placed and relatively quick solidifying (Spotnitz, 1995). The specific requirements for adhesives applied to living tissues, before and after curing, are summarised below.

BEFORE CURING	AFTER CURING
Sterilisable	Strongly bondable to tissues
Easy in preparation	Bio-stable union until wound healing
Viscous liquid or spray	Tough and pliable
Non-toxic	Resorbable after wound healing
Rapidly curable under wet, physiological conditions	Non-toxic
Reasonable cost	Not obstructive to wound healing or promoting wound healing

(Ikada, 1997)

The requirement for tissue adhesives to be liquids that solidify rapidly following application is particularly important, as this allows them to spread over the entire surface to be adhered and result in effective and close contact between the adhesive molecules and the tissue surface (Ikada, 1997). This binding to tissue surfaces, and the subsequent curing to a solid state, should also be unaffected by the presence of water (Otani *et al*, 1996). A further challenge is to couple adhesion properties with those of biodegradability, as tissue adhesives must always be re-sorbed in the body so that the wound edges can physically reunite for complete wound healing. This will then eliminate the need for post-operative removal, necessary for most mechanical fastening devices.

I-2 Current options for tissue fixation

I-2.1 Mechanical fastening

Sutures are the most common fixation method because they reliably close wounds until satisfactory healing occurs (Spotnitz *et al*, 1997). But mechanical techniques require time, post-operative removal and experienced handlers. Plus, as mentioned, many tissues are not capable of withstanding mechanical fastening techniques without sustaining further damage.

I-2.2 Synthetic adhesives

A large number of synthetic adhesives have been manufactured for industrial and consumer uses, but most are not appropriate as tissue adhesives. The major problems are the toxicity of adhesive ingredients, slow or no curing of the adhesive in the presence of moisture at body temperature, and insignificant *in vivo* biodegradability of cured adhesive (Ikada, 1997).

Cyanoacrylates are the only synthetic adhesives that meet most of the requirements for a tissue adhesive previously outlined (I-1). Their high bond strength has prompted some clinical application, but this is still associated with problems and the adhesive is not used internally.

Cyanoacrylate monomer viscosity is too low to allow the adhesive to adhere to tissues prior to polymerisation and the polymerisation process is often too rapid for convenient handling. The cured adhesive is then also often stiffer than the surrounding normal tissue (Otani *et al*, 1996). Cyanoacrylate polymers yield formaldehyde upon hydrolytic degradation *in vivo*, which is cytotoxic, and the presence of un-degraded polymer fragments can decrease the surface area available for collagenous union of wound edges and prevent fibroblast migration into the wound (Ikada, 1997).

Modified cyanoacrylates, such as octylcyanoacrylates, have had application in closing skin lacerations, but the wound edges must still be carefully approximated to keep the adhesive out of the wound, prevent toxicity and stop barrier formation (Rubin, 1998).

I-2.3 Fibrin sealant

Fibrin sealants are the most widely used and successful tissue adhesives to date (Jackson, 1996). They are based on the last step of the blood coagulation cascade and the formation of a network of cross-linked fibrin. Natural blood clot is an excellent adhesive for tissues, but occurs in insufficient amounts for urgent wound closure (Ikada, 1997).

Fibrin sealant is generally available as a freeze-dried powder in a kit, together with a thrombin, calcium chloride and aprotonin solution. These are used to prepare the two components of the adhesive which are mixed just before use (Taylor *et al*, 1992)

1. Sealant solution comprising fibrinogen, aprotonin and Factor XIII.
2. Activating solution comprising thrombin and calcium chloride.

I-2.3.1 Fibrin sealant – Chemistry

After mixing of the two-sealant components, the essential reactions within the adhesive are: coagulation, cross-linking and, finally, absorption of the clot material during wound healing within the body (Redl *et al*, 1980).

Fibrin sealants utilise thrombin, along with ionic calcium, to catalyse the polymerisation of fibrinogen into fibrin polymer and activate Factor XIII (FXIII, a cross-linking transglutaminase enzyme), to covalently cross-link the resultant gel (Sierra & Feldman, 1992). The fibrin network is the major component of the clot and Factor XIII is present in order to reinforce the polymer.

Fibrinogen, the main structural component of fibrin sealant, is proteolytically cleaved and converted to fibrin monomer by the action of thrombin. These monomers then aggregate into an unstable network linked by hydrogen bonds (Staindl *et al*, 1981). Factor XIII is also converted via the proteolytic action of thrombin (and the binding of calcium ions) to its active form FXIIIa, which replaces the non-covalent hydrogen bonds of the fibrin network, with covalent bonds by a process of transamidation (Aeschlimann & Paulsson, 1994). This cross-linking of the network increases the strength and stiffness of the fibrin gel, which becomes a semi-rigid, hemostatic, fluid-tight adhesive mass (Sierra, 1993). For an additional review of the properties of Factor XIII, see I-4.2.2.

I-2.3.2 Fibrin sealant - Physical properties

The physical properties of fibrin sealant can be considered in terms of both the fibrin-clot itself and its bonding capability.

The physical properties of a fibrin clot are dependent primarily on its fibrinogen concentration, though the structure of the sealant gel can also be altered by varying the thrombin and calcium concentrations, ionic strength and pH (Ikada, 1997; Sierra, 1993). *In vitro* studies have shown fibrin sealant coagulation time to be dependent on thrombin concentration, and that raising both the fibrinogen and thrombin concentrations results in clots of increased tensile strength (Redl *et al*, 1980; Nowotny

et al, 1980). However, thrombin concentration is generally reported to affect coagulation time alone and the fibrinogen content primarily governs clot strength (Spotnitz *et al*, 1997). These features may be used to modify sealant composition for particular clinical settings and sealant properties. However, it should not be assumed that these trends in mechanical properties hold when applied to all tissue types. When applied at a junction between blood vessels, fibrin sealant subjected to tensile and burst tests showed no increase in adhesive strength with increasing fibrinogen content or incubation time (Flahiff *et al*, 1982).

The tensile strength of the sealant material depends also on its degree of fibrin cross-linking, which increases the clots' rigidity (Redl *et al*, 1980). This cross-linking is produced by the FXIII component of the sealant, whose activity will in turn depend on the local thrombin and calcium concentrations. For an optimum rate of cross-linking, it is suggested that the CaCl concentration within the sealant should be greater than 5 millimoles per litre (Redl *et al*, 1980).

The FXIII content of the sealant (and the degree of fibrin cross-linking it provides) will affect the rate of clot fibrinolysis. Anti-fibrinolytic agents, such as aprotonin, are often included in sealant preparations to slow down the rate of clot degradation *in vivo*. However, high FXIII content is not necessarily advantageous as excessive sealant survival is not desirable, the clot should be re-sorbed *in vivo* in order to give way to native wound healing responses (Staindl *et al*, 1981).

The actual adhesive strength of fibrin sealant has yet to be fully characterised, with *in vitro* reports lacking complete descriptions of test parameters and control over test conditions (Sierra & Feldman, 1992). However, the bonding of human and rat skin by autologous and homologous fibrin sealant, as well as commercial fibrin adhesive, has shown the commercial preparation to be stronger. This is mainly due to having a higher fibrinogen concentration than it is possible to achieve with the autologous adhesive (Laitakari & Luotonen, 1989; Siedentop *et al*, 1988). An increased incubation time for the autologous adhesive also led to an increased adhesive strength. Shear adhesive strength was also shown to be directly proportional to fibrin concentration for cryoprecipitated fibrinogen preparations applied to split-thickness pigskin (Sierra & Feldman, 1992).

Published, quantitative bond strengths for fibrin sealant often vary due to sealant composition usually being unique to the author and there being variation in the test method and substrate choice. The comparison of fibrin sealant bond strength, with those of other adhesives, is difficult between experiments and direct comparisons within one experiment are rare. Chivers & Wolowacz (1997) directly compared the bond strength of a commercial fibrin sealant with those of a cyanoacrylate and a gelatin based-adhesive (GRF). They found that for the bonding of cartilage, bone and skin, the different adhesives gave bond strengths of approximately an order of magnitude difference, i.e. cyanoacrylates 1 MPa, GRF 0.1 MPa and fibrin 0.01 MPa. The bonding capabilities of fibrin sealant are also dependent on its site of application, with the water, collagen and fat content of a tissue all affecting adhesive strength (Ikada, 1997; Sierra & Feldman, 1992).

I-2.3.3 Fibrin sealant – Effect on wound healing

The influence of fibrin sealant on wound healing is controversial and often dependent on tissue type and how healing has been measured and assessed.

The components of fibrin sealant are intrinsically part of the normal events of hemostasis and wound healing (Sierra, 1993) and it is suggested that the clot formed by addition of sealant to a wound site may stimulate healing (Sclag & Redl, 1988). As well as the sealant holding the wound edges together, the fibrin network may act as a scaffold for migrating cells such as fibroblasts. Alternatively, the network may mechanically obstruct migration and proliferation of these cells (Ikada, 1997; Spotnitz *et al*, 1997).

The effects of fibrin sealant on wound healing may only be important during the first few days after trauma. During this stage fibrin, thrombin and factor XIII may facilitate the growth of new fibroblasts into a wound area and promote their laying down of collagen, whose amount and quality will help determine the firmness of the wound (Staindl *et al*, 1981). After this process the clot should be re-sorbed so that it does not become a barrier to repair. Too much sealant may lead to an excess of fibrous tissue and indeed delay normal cell migration and wound healing (Sierra, 1993). So in

assessing the influence of fibrin sealant on wound repair, the choice of end-point and evaluation is critical and far from universal.

Clinical studies have shown conflicting results. Fibrin sealant helped to decrease wound contraction when applied to skin grafts (Brown *et al*, 1992). Whereas, investigations using a skin incision wound suggested that the sealant provides greater wound strength only during the early stages of healing, and in fact delays healing over time (Jorgensen *et al*, 1987).

Despite these contradictions, the overall impression is that fibrin sealant can aid wound healing and that, in contrast to sutures, its application at a wound site does not seem to induce any *additional* tissue inflammation and reactivity.

I-2.3.4 Fibrin sealant – Clinical applications

Although not a substitute for sutures in terms of strength, fibrin sealants have been increasingly used in clinical settings, where their chief application is in hemostasis, rather than tissue adhesion. As currently formulated, fibrin sealant is not strong enough to hold skin edges together reliably, no matter the fibrinogen concentration (Spotnitz *et al*, 1997). If used in orthopaedic applications, the low bonding strength requires immobilisation of the joint and/or additional mechanical fixation for successful healing (Claes *et al*, 1980). So, in general, sealant bond strength is usually too low to give proper wound approximation until healing is complete and additional forms of stabilisation are often required (Ikada, 1997). However in certain clinical settings, the relatively low bonding strength of the sealant is compensated for by other desirable properties e.g. biocompatibility, biodegradation, quick application. In these situations the adhesive often makes for a beneficial adjunct to sutures (Spotnitz *et al*, 1997). Such as to help reduce suture number and scar formation (Sierra, 1993) and seal any gaps formed around suture holes.

The major uses of fibrin sealant have been thoracic and cardiovascular surgery, neurosurgery, and plastic surgery (Spotnitz, 1995). In cardiovascular surgery, fibrin sealants have been applied for blood-vessel anastomoses where use of sutures is

tedious and requires microsurgery. But again, bond strength was still significantly lower than that of the mechanical fixation method (Flahiff *et al*, 1992).

The use of an adjuvant (e.g. CaCl or aprotonin) or impregnating the sealant with a suitable vehicle to accelerate plug formation (e.g. gauze or collagen fleece) improves the bond strength of cryoprecipitate fibrin glue in hemostasis (Chang *et al*, 1992). These steps may be successful for a commercial preparation also. In neurosurgery, fibrin sealant again avoids microsurgery for nerve anastomosis and reduces the overall procedure time (Narakas, 1988; Sierra, 1993). Application in plastic surgery can improve cosmetic results and reduce scarring (Flemming, 1992).

There have also been applications in general, orthopaedic and paediatric surgeries (Spotnitz, 1995; Sierra, 1993). Use of fibrin sealant in orthopaedic surgery is discussed in I-3.6.

These applications of fibrin sealant have involved its use as a tissue-bonding or hemostatic agent, but it can also be used as a matrix for the delivery of active compounds. For example, growth factor for cartilage repair and endothelialisation of vascular grafts (Hashimoto *et al*, 1992; Greisler *et al*, 1992) and antibiotic release (Redl *et al*, 1983). Though the inclusion of antibiotics in a sealant preparation can increase coagulation time (Redl *et al*, 1980; Redl *et al*, 1983), this may be over come with the addition of exogenous Factor XIII and extra thrombin. It is the fibrinolysis of fibrin sealant *in vivo* that allows for a slow-release mechanism. Sealant breaks down to around 20% of its original mass by fibrinolysis over 72 hours in a wound, a rate that can be altered by the addition of anti-fibrinolytic agents (Spotnitz *et al*, 1997).

I-2.3.4 Fibrin sealant – Disadvantages

The main disadvantage of fibrin sealant is its' low bonding strength, but the concern that has led to its limited use in the USA has been the use of blood products in its preparation. Fibrin sealant can be produced from different sources, depending on how the fibrinogen is obtained. Commercial fibrin glue kits utilise fibrinogen from the blood of multiple human donors, which gives a risk of viral transmission. Several methods have been proposed as solutions including viral inactivation by heat or

nanofiltration, autologous donation or recombinant production of synthetic fibrin (Spotnitz *et al*, 1997). In the case of autologous production, the bond strength of the sealant produced will be dependent on the patients' plasma fibrinogen levels (Ikada, 1997).

Fibrin glues utilising bovine thrombin (to try and help reduce the risk of viral transmission) led to patients developing antibodies to the bovine thrombin and the development of bovine thrombin inhibitors, as well as co-immunisation to Factor V (Banninger *et al*, 1993). These antibodies can cross-react with human Factor V and have resulted in Factor V deficiency of sufficient severity as to cause bleeding complications (Jackson, 1996).

In terms of specific applications, fibrin sealant applied to blood vessels can adversely effect the local environment in terms of elevated thrombogenicity (Dumanian *et al*, 1995). Also, application into cartilage defects can lead to a host wound response that causes the formation of fibro-cartilage as repair tissue, rather than hyaline cartilage (Shapiro *et al*, 1993).

An additional disadvantage of fibrin sealants is the enzyme they use to provide structural integrity within the sealant, Factor XIII. Compared to other members of the transglutaminase family, FXIII has narrow substrate specificity, complex sub-unit configuration and complex activation requirements (see I-3.2.2) (Taylor *et al*, 1992).

I-2.4 Alternative adhesives

In response to the disadvantages associated with currently available tissue adhesives, research is continuing into the development of new formulations.

Non-biologic, photopolymerisable glues have been used to seal blood vessel anastomoses without augmenting thrombogenicity (Dumanian *et al*, 1995).

Hybridised systems are another alternative wherein an aqueous polymer solution is set to a gel when a cross-linking agent is added (Ikada, 1997). One such water-soluble polymer is gelatin, absorbable in the body, which has been used as a tissue adhesive when cross-linked by formaldehyde/glutaraldehyde activators (Stassano *et al*, 1994).

This adhesive is therefore free of blood components, but again utilises the toxic formaldehyde.

Another gelatin-based adhesive is a hydrogel of gelatin and poly (L)-glutamic acid (PLGA) cross-linked by a water-soluble carbodiimide. This has shown firm adhesion to mouse skin with a bonding strength higher than that of a commercial fibrin sealant (Otani *et al*, 1996). The advantages of this preparation is the lack of blood products, cheaper cost than fibrin sealant and a viscosity that allows retention at the site of application.

GXIII® (Unitaka Co, Japan) mimics fibrin sealant action and consists of thrombin and FXIII in a gelatin carrier. It produces local hemostasis by promoting fibrin formation from fibrinogen already at the wound site. This reduces the viral risk associated with fibrin sealant, though thrombin and Factor XIII are still present (Ikada, 1997).

The aim of this research is to develop a biological tissue adhesive that would meet those requirements listed in I-1, whilst avoiding the disadvantages associated with existing preparations. The bonding capabilities of this adhesive will be investigated initially at cartilage-cartilage interfaces, since it's primary application is initially intended for the field of cartilage repair. Therefore the structure, injury and repair of cartilage will now be considered.

I-3 Cartilage

I-3.1 Cartilage composition

Cartilage is termed dense connective tissue and contains almost entirely fibrous extra-cellular matrix materials with relatively few cells. Its capacity to function as a tough, wear-resistant, almost frictionless material depends on the concentration and organisation of its collagen, proteoglycan and water components to influence the tensile, compression and shear properties of the tissue (Buckwalter *et al*, 1990). Cartilage behaves as a fibre-reinforced composite material with two phases: a solid phase made up of macromolecules and cells, and a fluid phase of water and solutes (Mow *et al*, 1990). Cartilage matrix varies in appearance, composition and in the nature of its fibres, leading to the designation of the three chief types of cartilage: hyaline, fibrous (with much collagen) and elastic (with an elastin network) (Williams & Warwick, 1980). Articular cartilage refers to the hyaline cartilage situated at joint surfaces and is the main target for cartilage repair, as its injury is extremely common and can lead to significant reduction in the effective operation of the joint (see I-3.3).

I-3.1.1 Cartilage composition – Chondrocytes

The cells within cartilage are termed chondrocytes and occupy 1-10% of the tissue volume (Wirth & Rudert, 1996). Chondrocytes originate from mesenchymal stem cells and help maintain the mechanical properties of the tissue by replacing degraded matrix molecules (Temenoff & Mikos, 2000). Mature articular chondrocytes appear rounded, are completely encased within the matrix and do not form cell-cell contacts. Changes in matrix composition (through chemical or mechanical means) can either alter the synthetic function of chondrocytes, or damage them by impairing nutrition (Newman, 1998). Under normal circumstances, chondrocytes in mature cartilage rarely divide and cell numerical density decreases with age (Buckwalter *et al*, 1990; Temenoff & Mikos, 2000).

I-3.1.2 Cartilage composition – Collagen

The extra-cellular matrix of cartilage consists of densely packed collagen's and other fibrous proteins, surrounded by glycoproteins, proteoglycans and other substances produced and secreted by the relatively few cells present.

Collagens form around 50% of the dry weight of cartilage and whilst type II is the major form found (90-95% of the total), types VI, IX, X and XI are also present (Buckwalter *et al*, 1990; Newman, 1998).

Collagen II forms small, banded, inter-weaving fibrils and accompanying fibril formation is the oxidation of certain lysine and hydroxy-lysine residues by lysyl oxidase, with the reactive aldehyde groups produced forming specific, stabilising covalent cross-links between collagen chains (Darnell *et al*, 1990). Collagen II contains high amounts of bound carbohydrate groups, allowing for greater interaction with water than other collagen types (Temenoff & Mikos, 2000). The collagen fibre meshwork provides tensile strength for the tissue and entraps other macromolecules. Bound to the surface of the type II fibrils is type IX collagen, which helps anchor the collagen fibrils to proteoglycans and other matrix components.

I-3.1.3 Cartilage composition – Proteoglycans

The extra-cellular matrix of cartilage contains abundant proteoglycans and it is these which give cartilage its unique gel-like properties and resistance to deformations. They form around 30-35% of the dry weight of the tissue (Buckwalter *et al*, 1990) and have a half-life of 2-14 days (Wirth & Rudert, 1996). Proteoglycans are 95% polysaccharide, 5% protein and exist as multiple forms, aggregating and non-aggregating, large and small. Aggregating proteoglycans (aggrecans) are composed of multiple monomers of protein core filaments, to which are attached keratan sulphate and chondroitan sulphate glycosaminoglycans (Buckwalter *et al*, 1990).

Glycosaminoglycans (GAG's) are unbranched polysaccharides made from disaccharides of an amino sugar and another sugar. At least one component of the disaccharide has a negatively charged sulphate or carboxylate group, so that each

GAG chain contains a large number of negative charges, which tend to repel each other and other anions whilst attracting cations and facilitating interaction with water (Temenoff & Mikos, 2000).

In aggrecans, link proteins connect many monomers to hyaluronic acid, a long, negatively charged polysaccharide that forms viscous hydrated gels (Darnell *et al*, 1990). Aggrecans fill most of the extra-cellular matrix space and are responsible for resilience and stress distribution in cartilage through their ability to attract water. The aggregation of proteoglycans prevents their diffusion out of the matrix during loading and proteoglycan complexes bind to the surfaces of collagen fibres and link them together throughout the matrix. The smaller proteoglycans do not affect the physical properties of the tissue, but are thought to play a role in cell function and organisation of the collagen matrix (Temenoff & Mikos, 2000).

I-3.1.4 Cartilage composition – Non-collagenous proteins and glycoproteins

These form around 15-20% of the dry weight of the tissue (Buckwalter *et al*, 1990). Non-collagenous proteins' help to organise and stabilise the extra-cellular matrix as well as attach chondrocytes to it and stabilise the chondrocyte phenotype (Buckwalter *et al*, 1988).

I-3.1.5 Cartilage composition – Tissue fluid

Tissue fluid composes around 80% of the wet weight of cartilage and consists of water, in which are dissolved gases, metabolites and cations (to balance the negatively charged GAG's) (Temenoff & Mikos, 2000). Cartilage is an avascular tissue and its metabolism mainly anaerobic (Wirth & Rudert 1996), it is the exchange of tissue fluid with synovial fluid that provides the nutrients and oxygen required by the chondrocytes. In normal adult articular cartilage, the transport of these substances through the porous matrix occurs by diffusion, convection or a combination of the two processes (Mow & Rossenwaser, 1988).

Entrapment of the fluid through interaction with extra-cellular matrix components provides the tissue with its ability to resist compression and return to its normal shape after deformation. Under compression, tissue fluid flows out of the permeable collagen-proteoglycan matrix and when the load is removed, fluid flows back into the tissue (Newman, 1998). The low permeability of articular cartilage prevents the fluid from leaving the matrix too rapidly and protects the solid phase of the cartilage from the shock of high-impact loading (Mow & Rosenwasser, 1988). After prolonged and continuous loading, an equilibrium is reached and the fluid flow ceases. In this case, the load is now supported by the solid phase of the tissue (Newman, 1998; Mow & Rosenwasser, 1988).

I-3.2 Cartilage organisation

Of the three main types of cartilage, fibrous and elastic cartilages are relatively unvarying tissues, whereas hyaline cartilage can exhibit a range of appearances depending on age and location. In humans, costal, nasal, tracheobronchial, some laryngeal and most articular cartilages are hyaline, but they differ in size, shape and arrangement of cells, fibres and proteoglycan composition (Williams & Warwick, 1980). In this study, we will be concerned with hyaline cartilage and specifically costal and articular cartilages. A diagram of collagen fibre and proteoglycan organisation and composition in hyaline cartilage is shown in Figure I-1.

Hyaline cartilage cells are relatively flat near the perichondrium (fibrous covering surrounding cartilage, except at synovial surfaces) and more rounded deeper in the tissue. They are often in groups of two or more where they have a straight outline where opposed to each other, but a rounded contour in general. There is often an encapsulating layer of proteoglycans immediately surrounding the cell and outside this there are thin collagen fibres, often arranged in a basket-like network around one or more cells, grading into a coarser fibre domain between cells. In costal cartilage, cells are large and the matrix is prone to calcification (Williams & Warwick, 1980).

Articular hyaline cartilage covers articular surfaces and is generally classified into zones of organisation, according to distance from the joint surface. These zones differ

in composition and are termed superficial, transitional or middle, deep and calcified (see Fig I-2).

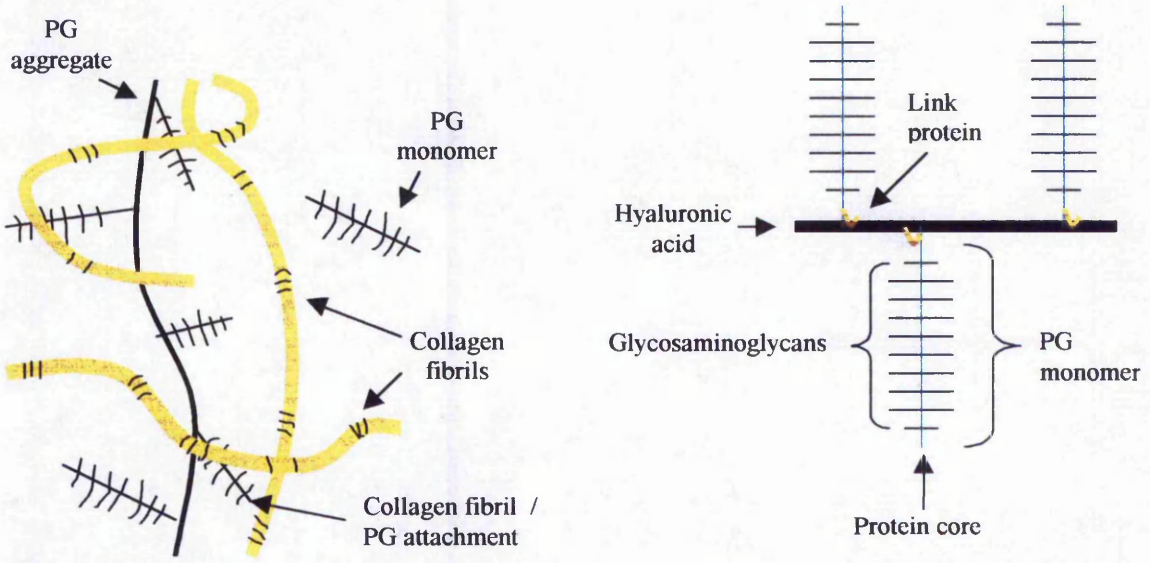
The superficial zone is the thinnest and consists of an acellular sheet of collagen fibres covering the joint (Temenoff & Mikos, 2000). Below this are flattened, inactive chondrocytes with their long axis parallel to the articular surface and the matrix here contains more collagen and less proteoglycan than other zones, along with much fibronectin and water (Newman, 1998). This combination imparts tensile strength, useful for resisting shear forces and important for the compressive strength of the tissue (Temenoff & Mikos, 2000).

The transitional or middle zone contains spherical chondrocytes and larger collagen fibres, randomly aligned (Newman, 1998; Buckwalter *et al*, 1990). The matrix contains more proteoglycan and less water than the superficial zone (Temenoff & Mikos, 2000).

As the tissue progresses into the deep zone its cells align into columns, perpendicular to the articular surface (Buckwalter *et al*, 1990). The collagen fibres have a similar orientation and insert into, and past, the tidemark that indicates the start of the calcified tissue (Newman, 1998; Temenoff & Mikos 2000). Calcified tissue is the transition between soft and hard tissue and the inserting collagen fibres help fix the two together (Buckwalter *et al*, 1990; Newman, 1998). Any chondrocytes in this zone are small and in some places completely surrounded by calcified extra-cellular matrix, indicating very little metabolic activity (Temenoff & Mikos 2000).

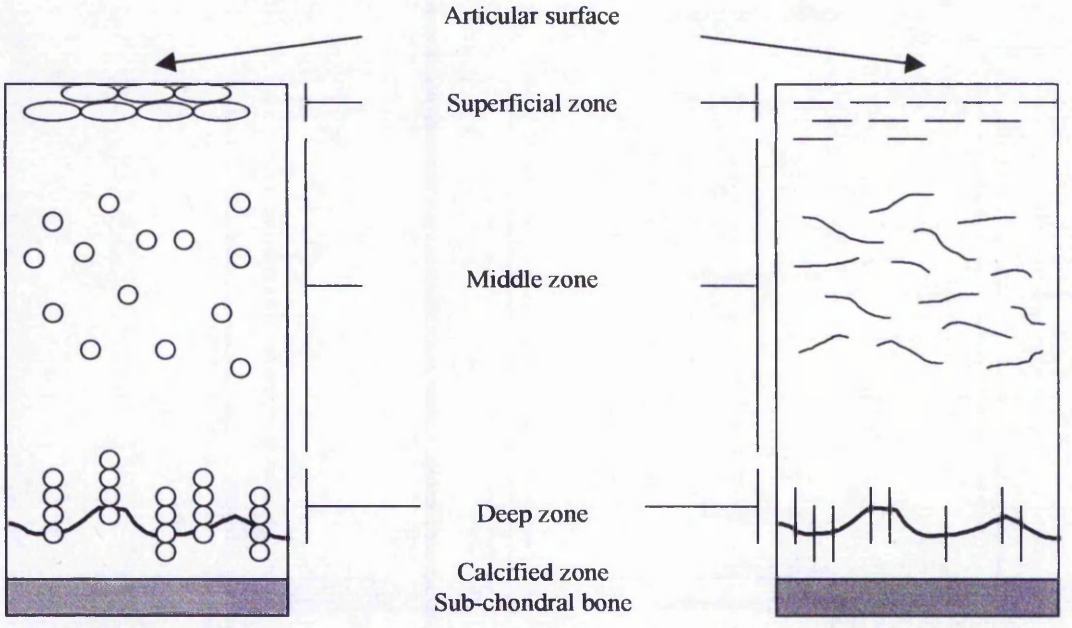
Articular cartilage also consists of matrix regions, termed pericellular, territorial and inter-territorial, which differ in composition, organisation and function depending on distance from the cells (similar to that described for non-articular hyaline cartilage). The pericellular region directly around the cells is almost exclusively non-collagenous binding proteins and abundant proteoglycans (Temenoff & Mikos, 2000; Newman, 1998). Outside this region is the territorial matrix containing a thin web of collagen fibrils and these two regions provide the means for chondrocyte attachment to the extra-cellular matrix, and protection of the cells during loading. Further away from the cells, in the inter-territorial matrix, collagen fibril diameter increases and their orientation is according to distance from the joint surface and zonal classification. This region is primarily responsible for the mechanical characteristics of the tissue.

Figure I-1. Schematic diagram of the composition of hyaline cartilage in terms of collagen fibre and proteoglycan (PG) organisation and PG aggregate composition



A) Collagen fibrils and proteoglycans **B) PG aggregate composition**

Figure I-2. Schematic diagram of the orientation of a) chondrocytes and b) collagen fibres in articular hyaline cartilage



A) Chondrocytes **B) Collagen fibres**

I-3.3 Cartilage injury and intrinsic repair

The response of cartilage to injury depends on the nature and extent of the injury (Buckwalter *et al*, 1988).

In general, the repair of defects in cartilage is rarely successful in the long term due to several factors that limit the tissues' response to injury:

1) A lack of blood vessels within the tissue which prevents new cells from entering the wound site, haemorrhage, fibrin clot formation or inflammation
2) A lack of undifferentiated cells that can repair the tissue, preventing the initiation of a healing response as seen in other tissues
3) Chondrocytes being encased and immobilised in a dense, strong collagen-proteoglycan matrix, giving limited capacity for migration and proliferation.

In other tissues, blood vessel damage leads to fibrin clot formation and the initiation of an inflammatory response that includes inflammatory cell migration to the defect site. Injured cells and platelets release mediators to promote this response and the inflammatory cells clear the necrotic tissue and release stimulants of migration of mesenchymal cells that invade the fibrin clot, proliferate, differentiate and synthesise new matrix (Mow *et al*, 1991).

Cartilage injury can be divided into three general classes of matrix disruption, partial thickness defects and full-thickness defects.

Matrix disruption occurs mainly from blunt trauma, as loading occurs too quickly for fluid movement through the collagen matrix and the matrix macromolecular framework sustains a greater share of the stress (Buckwalter, 1998). If the chondrocytes remain viable and the collagen network intact, the cells are able to increase synthesis of matrix molecules and repair the tissue (Temenoff & Mikos, 2000). If the injury exceeds the capacity of the chondrocytes to restore the matrix, the damage is irreversible. So damage that causes massive loss of proteoglycans, disrupts the collagen network, or causes cell death is usually not repaired with normal cartilage matrix (Buckwalter *et al*, 1988).

Defects in articular cartilage are classified as partial or full thickness, depending on whether they remain within the cartilage or penetrate through to the sub-chondral bone. They can thus be designated as chondral and osteochondral injuries.

Partial-thickness defects have no access to blood cells, therefore there is no fibrin clot formation, little evidence of cell migration and these defects tend not to heal (Hunziker, 1999a; Buckwalter *et al*, 1988). Chondrocytes near the lesion may show some signs of proliferation and synthesise new matrix, but they don't migrate into the lesion and any new matrix formed fails to fill the defect (Buckwalter, 1990).

Partial-thickness defects are analogous to the clefts and fissures seen in cartilage during the early stages of osteoarthritis (Hunziker, 1999a). In fact, damage to the articular cartilage of the knee is regarded as being a precipitating event in the accelerated onset of osteoarthritis (Gillogoly & Newfield, 2000).

Full-thickness cartilage defects lead to a vascular repair response, as they penetrate through to the sub-chondral bone. But the defect fills with a fibro-cartilaginous repair tissue that lacks the composition, structure and durability of normal cartilage (Buckwater *et al*, 1988). This spontaneous repair progresses through the stages associated with repair of other tissues: fibrin clot formation, mesenchymal cell migration, differentiation and resorption of the clot, and repair tissue being laid down (Hunziker, 1999a). But the fibrous tissue eventually fragments and often disintegrates. (Buckwalter, 1998). Points of discontinuity with the original host tissue are seen at the defect edges and no true integration occurs as the collagen fibrils of each component fail to mingle. This bonding problem represents a major stumbling block encountered in chondral repair systems (Hunziker, 1999b).

Another modulating factor that can influence the cartilage healing response is the age of the organism. With increasing age, articular cartilage undergoes structural, molecular, cellular and mechanical changes that seem to increase its vulnerability to degeneration and decrease its ability to repair or restore itself (Buckwalter, 1999). With age, the wound healing and regenerative capacities of cartilage decrease (Verwoerd-Verhoef *et al*, 1998). Articular chondrocytes from skeletally immature animals have a greater ability to proliferate and synthesise larger proteoglycan molecules (Newman, 1998).

I-3.4 Cartilage repair techniques

To overcome the inherent deficiencies of cartilage for repair, various techniques have been developed to try and manipulate the tissue environment. These have mainly focused on articular cartilage defects and the correcting of one of the two main shortcomings of the tissue, by bringing in cells capable of chondrogenesis, and/or, facilitating access to the vascular system (Newman, 1998).

A problem in the evaluation of cartilage repair techniques has been the assessment of the quality of the repair tissue formed. Many studies do not include long-term follow-up, or mechanical and histological evaluation. Appropriate comparisons between repair techniques are also scarce.

I-3.4.1 Mechanical techniques for promoting cartilage repair

A method commonly employed to stimulate repair in partial, and full-thickness, injuries has been penetration of the sub-chondral bone. This is achieved by drilling, or abrasion, to provide blood elements which will form a fibrin clot in the defect and allow access to mesenchymal stem cells that can differentiate into cartilage (Gillogly & Newfield, 2000). However, as with the intrinsic repair seen in full-thickness defects, the repair process leads to fibro-cartilage formation. This fails to duplicate articular cartilage composition and mechanical properties and fails with time (Buckwalter & Mankin, 1998). The technique gives inconsistent results and often, recurrent symptoms (Gillogly & Newfield, 2000).

Other mechanical treatments include: osteotomy and joint realignment to alter the contact stress on damaged regions and joint distraction to decrease contact stress by the use of an external fixator structure (Buckwalter & Mankin, 1998). But none of these approaches has stimulated regeneration of normal articular cartilage in skeletally mature humans.

I-3.4.2 Tissue transplantation

An alternative to stimulating the intrinsic repair of damaged cartilage is the technique of tissue transplantation. In which, the defect is filled with cells and/or tissue, capable of restoring the articular surface or duplicating its mechanical characteristics.

As well as transplanting chondral and osteochondral fragments, tissues such as periosteum and perichondrium have been used for graft material, along with *in vitro* generated scaffolds seeded with chondrogenic cells (Mow *et al*, 1991). In general, whichever material is used, it should:

1) Contain a cell population which can meet the metabolic demands of restoring a functional extra-cellular matrix
2) Fill the volume of defect to allow continuity across the joint surface
3) Have a method for adequate internal fixation that withstands the forces of joint movement and doesn't interfere with the repair process (Grande *et al*, 1999)

I-3.4.2.1 Chondral and osteochondral grafts

Chondral and osteochondral grafts have the advantage of placing chondrocytes into the defect, which are already surrounded by fully formed cartilage matrix. The transplanted tissue may be in the form of an autograft or an allograft

Autografts are appropriate only for smaller defects, as there is a limited availability of sites for harvest. Where as allografts can be prepared in greater size and be taken from the same location on the donor as the recipients' lesion. Thus providing a good match in terms of contour and thickness (Newman, 1998). Allografts carry a risk of viral transmission and possible immunogenicity. But in autografts, the age of the donor tissue is fixed where as allografts can be taken from younger individuals (Newman, 1998).

With both graft types, consistent healing of the chondral part of the graft to the adjacent cartilage has not been demonstrated (Buckwalter & Mankin, 1998). Problems of integration and long-term stability have been raised and the mechanical processes

of harvesting and pressing grafts into the defect site can reduce chondrocyte viability within the graft tissue (Hunziker, 1999a). There is also concern for the long-term effect on the remaining surrounding tissue at the donor site.

Articular cartilage is considered immunologically privileged because its avascularity isolates it from the immune system. But isolated allograft chondrocytes do contain surface transplantation antigens and will provoke an immune response if placed in a full-thickness defect and exposed to the recipients' vascular system. Allograft bone also possesses immunogenic cells (Newman, 1998).

I-3.4.2.2 Periosteum and Perichondrium

Periosteum and perichondrium are used in cartilage repair as they are rich in multi-potent, osteo-chondral progenitor cells (Boyan *et al*, 1999). Periosteal and perichondrial grafts replace damaged articular cartilage with a soft tissue of chondrogenic potential, whose cells have the capacity to form a hyaline-like cartilagenous matrix, similar to the native tissue (Mow *et al*, 1991). The technique tends to produce better results with younger patients, as the population of cells that can form cartilage declines with age. Plus, perichondrium is only available at the sternal margin of ribs and so there is a limited supply (Buckwalter & Mankin, 1998). Complete restoration and long-term stability has not yet been achieved with this method and attempts to prevent detachment with suturing and glues have proved difficult (Hunziker, 1999a).

I-3.4.2.3 Cell transplantation

Cell transplantation involves the harvesting of cells with chondrogenic capacity (e.g. chondrocytes, mesenchymal stem cells), their *in vitro* culture and subsequent implantation into defect sites. As with soft tissue grafts, the matrix these cells produce should then closely resemble normal hyaline cartilage.

Chondrocytes are relatively easy to obtain but as cell culture time increases, the cartilage they form is increasingly fibrous in nature (Temenoff & Mikos, 2000). In that the quantity of type II collagen present is greatly reduced and type I collagen fibres predominate. The implanted chondrocytes may also not restore the correct matrix in terms of zonal orientation, as their *in vitro* culture may proliferate one zonal sub-population over another (Grande *et al*, 1999).

The major problem of this technique is the retention of cells in the defect site and cell transplantation is often used alongside other repair methods, such as periosteum (Brittberg *et al*, 1994) and scaffolds (see I-3.4.2.4), to solve the problem. Autologous-chondrocyte transplantation, for full thickness defects of the knee, is one cartilage repair method that has been studied long-term (2-9 years, Petterson *et al*, 2000). Periosteum was used to retain the cells in the defect site, fixed in place by sutures and fibrin sealant, and good clinical outcome was observed with the technique, which has now progressed to clinical trials to assess its effectiveness versus traditional fibrocartilage repair techniques.

A further general disadvantage of the cell-transplantation method is that it requires two surgical interventions, for harvest and re-implantation (Hunziker, 1999a; Buckwalter & Mankin, 1998)

I-3.4.2.4 Synthetic and biological matrices

Matrices are implanted into cartilage defect sites to act as scaffolds and retain implanted cells. In the case of chondrocytes this is especially important as the cells can then be maintained in a 3-dimensional network, which allows them to retain their rounded, chondrocyte phenotype.

Desirable properties for the matrix are that it needs to be biocompatible, mechanically stable, amenable to rapid remodelling and allow adequate adhesion to the defect surface (whilst facilitating integration) (Hunziker, 1999b). The use of a mouldable matrix also allows any size and shape defect to be filled.

A variety of biologic and non-biologic materials have been proposed as suitable scaffolds such as collagens, fibrin, hyaluronan, agarose, carbon fibre,

polytetrafluoroethylene and polyester (Buckwalter & Mankin, 1998; Bahforth *et al*, 1998).

The most attention for a natural scaffold has been on collagen, which can be remodelled by cellular enzymes and has proper cues to stimulate collagen production by transplanted cells (compared to other scaffold types). The use of a collagen gel hardened by cultured allogenic chondrocytes, implanted into full-thickness articular cartilage defects in rabbits, showed good repair up to 6 months (Kawamura *et al*, 1998). Frenkel and co-workers (1997) investigated chondrocyte transplantation, using a collagen bi-layer matrix comprising a dense collagen layer, for contact with the sub-chondral bone, and a porous matrix for seeding with chondrocytes. Over a 6-month period of observation, hyaline-like repair tissue was observed whose glycosaminoglycan and collagen II content were near normal. The use of the dense layer was thought to prevent fibroblast in-growth from below the defect, yet allow growth factor influx that promoted cell growth.

A natural glycosaminoglycan polymer such as a hyaluronan has the ideal physical, chemical and biological properties to serve as a matrix. It is found in the extra-cellular matrix of most tissues and forms viscous aqueous gels. These properties have led to its use as a carrier for the sustained systematic delivery of proteins, peptides and traditional pharmaceuticals (Radomsky *et al*, 1998).

But producing natural polymers in large amounts may not be feasible and there may be no guarantee of pathogen removal (Temenoff & Mikos, 2000).

Synthetic matrices can be tailored to be degradable (to allow tissue growth in the construct and eliminate the need for second surgery) and to include growth factors (see I-3.4.3). Polyglycolic acid (PGA), poly-l-lactic acid (PLLA), poly-dl-lactic-co-glycolic acid (PLGA) and poly-(α-hydroxy esters) are examples of such that can be degraded by hydrolysis.

Grande *et al* (1997) evaluated a range of matrix scaffolds for articular cartilage grafts. They cultured chondrocytes on various graft materials to see the effect on cell viability and matrix synthesis. Bio-absorbable polymers such as PGA, enhanced proteoglycan synthesis, whereas collagen matrices stimulated collagen synthesis.

Therefore, the choice of carrier material is important for chondrocyte viability and the activity and quality of the repair tissue.

The main disadvantage of scaffolds is that they require an operation for implantation, but they can be used in conjunction with other repair techniques and as a method for the delivery of growth factors and cells into the defect site.

I-3.4.3 Growth factors

Growth factors may be applied to a defect to stimulate cartilage formation and can be combined with other treatments. If no growth factors are included, then any repair cells in the defect will be relying on unpredictable stimulation from the wound environment (Hunziker, 1999a). The use of these molecules may also cause undesirable, multiple effects though (Buckwalter & Mankin, 1998).

Various studies have analysed the influence of bioactive molecules on cartilage synthesis and degradation (Newman, 1998).

Bone morphogenetic protein (BMP-7) was found to enhance the proliferation of cells with a chondrocyte phenotype in an articular environment (Mattioli-Belmonte *et al*, 1999). In this case N,N-dicarboxymethyl chitosan was used as the delivery agent and the treatment shown gave a higher number of cells with chondrocyte features than the control.

TGF-β_1 has the capacity to regulate cartilage proteoglycan metabolism in bovine cartilage cultures (Morales & Roberts, 1988). PDGF is a chemo-attractant for neutrophils, monocytes and fibroblasts and stimulates them to synthesise fibronectin and collagenase. IGF can enhance cartilage matrix synthesis and decrease proteoglycan degradation (Mow *et al*, 1991).

FGF and insulin, used with collagen gel scaffolds seeded with lapine articular chondrocytes, tripled the rate of cell turnover and doubled the glycosaminoglycan content of seeded implants. Their addition created the most biologically active implant with the greatest metabolic activity and least collagen degradation (Toolan *et al*, 1996). Basic FGF is a mitogen for chondrocytes and can stimulate cartilage matrix

production by the cells, as well as inhibit their terminal differentiation (which leads to decreased proteoglycan synthesis) (Mow *et al*, 1991). In addition, basic FGF, in a sodium hyaluronate gel, has been shown to give increased bone formation and earlier restoration of mechanical strength at fracture sites (Radomsky *et al*, 1998).

I-3.5 Integration between native and repair tissue at cartilage defect sites

A final hurdle to full cartilage regeneration is the integration of any newly formed repair tissue with the existing cartilage. Without this integration, forces that occur at the interface could have deleterious long-term effects on the replacement tissue (Temenoff & Mikos 2000). Uninterrupted continuity between the repair and native tissue needs to be established from the onset and subsequently maintained. This is essential for the proper biomechanical functioning of the cartilage as a whole. If discontinuities occur at any point along the interface, then forces generated during loading cannot be transmitted smoothly. This will lead to stress zones forming, which are vulnerable to enzymatic degradation (Hunziker, 1999b).

A clinical limitation of cartilage repair technologies has been the secure anchoring or attachment of repair materials in defect sites. Since one of the properties of articular cartilage is its low-friction and anti-adhesive surface, the adhesion between native and implanted material is usually inadequate (Jurgenson *et al*, 1997). The best preparation for repair may be compromised unless there is a simple and effective method for delivering and holding repair tissue securely in the defect environment (Grande *et al*, 1999). Sutures may be used to hold transplanted tissue in defect sites, but these can then cause defects to form at the suture sites, glues are an alternative but finding an appropriate one is still an unmet goal (Hunziker, 1999a).

The overall aim of this work was to develop a biological adhesive for use in cartilage repair that would help to reduce these problems of integration between native and repair tissue.

I-3.6 Cartilage repair and tissue adhesives

Most biological adhesives currently used in orthopaedic surgery are fibrin-based, but none has given satisfactory results (Jurgensen *et al*, 1997). One of the reasons for this has been that the introduction of a fibrin clot into cartilage defect sites has led to the stimulation of fibro-cartilage as the repair material (Aeschlimann & Paulsson, 1996). Another is the general low bond-strength of fibrin sealant not being suitable for use in high load-bearing joint regions.

Fibrin sealant can aid the attachment of osteochondral fragments, but the area requires additional forms of stabilisation (Sclag & Redl, 1988). The adhesiveness of cartilage to cartilage, and cartilage to bone was found to be very low using the sealant. Kaplonyi and co-workers (1988) found that the sealant had advantages over internal fixation (no further damage to the tissue, removal operation unnecessary), but again, only provided contact stabilisation.

The bond strength achieved with use of fibrin sealant on skin wounds was also shown to be achieved in the attachment of periosteum to cartilage (Orr *et al*, 1999). But where periosteum patches are used in cartilage repair, they still tend to be sutured in place for maximum adhesion, with fibrin glue merely being used to seal the suture holes.

In terms of quality of repair tissue formed, Niedermann and co-workers (1985) used fibrin glue to attach periosteal grafts in the knee and arthroscopic examination showed new cartilage formed. No histological examination of the nature of the repair tissue was performed however. Meniscal repair using fibrin sealant and endothelial cell growth factor showed an organised fibrous tissue formed, which changed to cartilaginous tissue over time. But this tissue was still different (in terms of histology and gross appearance) from normal meniscal tissue (Hashimoto *et al*, 1992). Brittberg and co-workers (1997) found that fibrin sealant (applied to an osteochondral defect in the rabbit knee) actually impaired the natural repair of the defect, with the sealant possibly functioning as a hemostatic barrier, reducing the endogenous fibrin response.

Using fibrin glue as a scaffold for implanting chondrocytes into a cartilage defect also failed because the glue did not offer enough mechanical support to the cells to maintain its function as a 3-d scaffold (Van Susante *et al*, 1999).

The ultimate aim of this work was to produce a biological adhesive for application in cartilage repair, based on the protein cross-linking enzymes transglutaminases, which would lead to greater success in bonding cartilage repair materials than existing preparations.

I-4 Transglutaminases

Transglutaminases (Enzyme commission system of classification 2.3.2.13) are a group of enzymes that catalyse the post-translational modification of proteins referred to as the R-glutaminyl-peptide, amine-γ-glutamyl transferase reaction. This is the post-translational exchange of primary amines for ammonia at the γ carboxamide group of glutamine residues.

When the amine derives from peptide bound lysine, the cross-linking of proteins, either intramolecular or intermolecular, occurs via ε(γ-glutamyl) lysine bonds. The resulting bonds are stable, covalent and increase the resistance of tissues to chemical, enzymatic and physical degradation (Greenberg *et al*, 1991).

The catalytic mechanism of transglutaminases (TG's) proceeds through an acyl-enzyme intermediate and is driven by the release of ammonia and its subsequent protonation under biological conditions (Lorand & Conrad, 1984). Other acyl acceptor groups may be the primary amino groups of naturally occurring polyamines, such as putrescine and spermidine, or, when an amine is not available, the acyl-enzyme intermediate may react with water to yield a glutamic acid residue (Folk, 1980). The reaction scheme of transglutaminase is summarised in Fig I-3.

The covalent cross-links resulting from transglutaminase activity were thought to be irreversible in most situations and resistant to most proteases (Lorand and Conrad, 1984). But the hydrolysis of the ε(γ-glutamyl) lysine isopeptide by tissue transglutaminase and Factor XIII has subsequently been demonstrated (Parameswaran *et al*, 1997).

Besides playing a structural role, transglutaminase cross-linking has been shown to modulate the biological activity of signalling proteins such as IL-2, TGFβ1 and midkine, and thereby to have a profound effect on cells (Aeschlimann & Thomazy, 2000; Kojima *et al*, 1993; Kojima *et al*, 1995; Kojima *et al*, 1997).

The number of proteins capable of acting as glutaminyl substrates for TG's is restricted, due mainly to the primary structure and conformation around the proposed residue. However, most proteins are able to contribute ε-amino groups from lysine residues to the cross-linking reaction (Aeschlimann & Paulsson, 1994). Glutaminyl specificity also differs between TG's, being more stringent for Factor XIIIa than for tTG. Close alignment of the glutamine and primary amine substrate can promote the cross-linking reaction, e.g. alignment of fibrin molecules after cleavage of fibrinogen, which promotes Factor XIIIa-mediated cross-linking between the fibrin γ chains followed by formation of α-chain multimers (Greenberg *et al*, 1991).

I-4.1 Transglutaminase family

In vertebrates, TG's form a large protein family and have a wide distribution amongst tissues and body fluids. Enzymes with a similar function to vertebrate TG's have also been found in invertebrates, plants and microorganisms (Aeschlimann & Paulsson, 1994).

In mammals the TG's so far characterised are genetically distinct, have a reactive cysteine at their active site which shares a common amino-acid sequence (Y-G-Q-C-W-V), are dependent on Ca for their activity and are found both in the intra-cellular and extra-cellular environment (Griffin & Verderio, 2000). The binding of Ca^{2+} ions leads to the exposure of the active-site cysteine residue and this requirement of TG's in higher animals, for calcium ions for activation, is less stringent in micro-organisms and plants (Greenberg *et al*, 1991).

In humans, several TG genes have been identified so far and their products found to cross-link proteins in different biological processes (Aeschlimann & Paulsson, 1994; Aeschlimann *et al*, 1998). The physiological functions of the various transglutaminases are still not fully understood, with the exception of the plasma transglutaminase, Factor XIII. Members of the transglutaminase family and their actual or hypothetical physiological roles are summarised in Table I.1.

Fig I-2. Reactions catalysed by Transglutaminase

A) $Gln\text{-}CO\text{-}NH_2 + RNH_2 + TG + Ca^{2+} \rightarrow Gln\text{-}CO\text{-}NHR + NH_3$

B) $Gln\text{-}CO\text{-}NH_2 + H_2N\text{-}Lys + TG + Ca^{2+} \rightarrow Gln\text{-}CO\text{-}NH\text{-}Lys + NH_3$

C) $Gln\text{-}CO\text{-}NH_2 + HOH + TG + Ca^{2+} \rightarrow Gln\text{-}CO\text{-}OH + NH_3$

D) $Gln\text{-}CO\text{-}NH_2 + {}^{+}H_3N\text{-}R\text{-}NH_3{}^{+} + H_2N\text{-}CO\text{-}Gln + TG + Ca^{2+} \rightarrow$

$Gln\text{-}CO\text{-}NH\text{-}R\text{-}NH\text{-}CO\text{-}Gln + 2NH_3$

A – Acyl transfer reaction, B – Cross-linking reaction between Gln and Lys of proteins, C – Deamidation, D – Cross-linking via bifunctional amines (e.g. putrescine)

Table I-1. Transglutaminase nomenclature and distribution

Name	Alternative designations	Location
Factor XIII	Plasma TG, Laki-Lorand factor, Fibrin-stabilising factor, Fibrinoligase	Plasma, Platelets, Macrophages, Monocytes, Hepatocytes, Placenta
Keratinocyte Transglutaminase	TG_K, Type I-, Particulate-TG	Stratified squamous epithelium
Tissue Transglutaminase	TG_C, Type II-, Cytosolic-, Liver-, Erythrocyte-, Endothelial- TG	Most cells and tissues
Epidermal Transglutaminase	TG_E, Type III-, Callus-, Bovine snout-, Hair-follicle- TG,	Epidermis, Dermal fibroblasts, Inner and outer root sheaths of hair follicles
Prostate Transglutaminase	TG_P, Dorsal prostate protein 1, vesiculase, major androgen-regulated prostate secretory protein	Semen
Erythrocyte band 4.2	B4.2	Erythrocytes

I-4.2 Types of transglutaminase

Several transglutaminases can be present in the same organism performing different functions, such as in humans, where the enzymes co-exist and appear to be involved with the cross-linking of different specific substrates at different locations within the body.

I-4.2.1 Erythrocyte Band 4.2, Keratinocyte and Epidermal transglutaminases, TG_x, and Prostate transglutaminase

The erythrocyte membrane-protein Band 4.2 is a 77 Kda structural component of the cytoskeletal network, underlying the red blood cell membrane. It is a catalytically inactive member of the transglutaminase family, due to the substitution of an alanine for the active site cysteine (Aeschlimann *et al*, 1994).

Keratinocyte transglutaminase (TG_K, Type I TG) and epidermal transglutaminase (TG_E, Type III TG) are expressed in different stages of epidermal differentiation and cross-link the structural proteins which form the cornified cell envelope (Aeschlimann & Thomazy, 2000). A further novel transglutaminase, designated TG_x, has also been identified in human keratinocytes (Aeschlimann *et al*, 1998).

Prostate transglutaminase (TG_P, Type IV TG) is an androgen-regulated protein involved in semen coagulation and its expression is restricted to prostate tissue (Dubbink *et al*, 1996).

I-4.2.2 Factor XIII, plasma transglutaminase

Factor XIII is the last zymogen to become activated in the blood coagulation cascade in vertebrates (Lorand & Conrad, 1984). It is a plasma protein which circulates in the blood as a tetramer of 'a_2b_2' (Mr ~ 320,000), consisting of two catalytic 'a' sub-units (Mr ~ 75,000 each) and two non-catalytic 'b' sub-units (Mr ~ 80,000 each). The 'b'

sub-unit function is not clear, but is thought to help stabilisethe catalytic 'a' sub-unit (Ichinose *et al*, 1990).

During activation, thrombin proteolytically cleaves activation peptides from the Factor XIII 'a' sub-units. This proteolysis is not sufficient to activate the enzyme, as the binding of Ca^{2+} is still required to unmask the active site cysteine and cause the tetramer to dissociate (Curtis *et al*, 1974). This reversible dissociation gives rise to a catalytic dimer a'_2 (termed Factor XIIIa) and a non-catalytic dimer b'_2. The presence of fibrinogen, both in fibrin sealant and natural blood clots, reduces the calcium concentration required for b chain dissociation to physiological levels (Greenberg *et al*, 1991).

In vivo, Factor XIII is highly specialised for the blood-clotting reaction and rapid cross-linking of fibrin chains, despite having many substrates under *in vitro* conditions (Mosesson, 1997). Factor XIII catalyses cross-linking of fibrin γ chains to form characteristic γ-chain dimers, followed by covalent stabilisation of the fibrin α and γ chains and α-chain polymerisation (Shainoff *et al*, 1991; Chen & Doolittle, 1971). Factor XIII is associated with fibrinogen when circulating in plasma (Hornyak & Schafer, 1992; Achyuthan *et al*, 1995) and also cross-links α_2-plasmin inhibitor to the α-chain of fibrin, protecting the fibrin clot from fibrinolysis by plasmin (Tamaki & Aoki, 1982). This reaction occurs faster than the other cross-linking mechanisms.

Polymerising fibrin polymers increase the rate of thrombin proteolysis of Factor XIII by providing binding sites for both enzymes. This helps to regulate that FXIIIa is formed after fibrin and also limits the formation of FXIIIa in plasma (Greenberg *et al*, 1987).

A platelet form of the Factor XIII enzyme also exists, as a catalytic dimer 'a_2'' derived by thrombin activation on its two sub-unit zymogen 'a_2'. Isolated 'b_2' from plasma enzyme may be combined with platelet enzyme, in the absence of Ca^{2+}, to give a four sub-unit molecule that is indistinguishable from the plasma enzyme (Chung *et al*, 1973). This pro-transglutaminase from platelets is activated by thrombin at a faster rate than the zymogen from plasma.

As well as fibrin, other proteins found in blood plasma and/or the vascular wall are Factor XIII substrates, such as fibronectin (Fesus *et al*, 1986) and vitronectin (Sane *et al*, 1988), which may help contribute to wound healing processes, matrix assembly

and cell-matrix interactions. Exogenous fibronectin is susceptible to FXIIIa cross-linking at sites of matrix assembly (Barry & Mosher, 1988), which enhances the formation of extra-cellular matrix (ECM) and may facilitate wound healing by providing additional mechanical resistance to wounded tissue (Aeschlimann & Paulsson, 1994). Factor XIII cross-links fibronectin to both fibrin α-chains and to collagen and the cross-linking of fibronectin to fibrin by FXIIIa has been shown to be required for maximal cell adhesion to a fibronectin-fibrin matrix (Corbett *et al*, 1997). In addition, Factor XIII has been demonstrated to bond to the surface of cultured fibroblasts and mediate the cross-linking of cellular and plasma fibronectin to the ECM (Barry & Mosher, 1990; Barry & Mosher, 1989; Quade & McDonald, 1988).

I-4.3 Tissue transglutaminase (tTG)

Tissue transglutaminase (tTG, also known as Type II transglutaminase and TG_c) is a multi-functional enzyme involved in disparate biological processes (Aeschlimann & Thomazy, 2000). It has been characterised in many cells and tissues and exists as a monomer of molecular weight ~ 75 - 80 Kda (Ichinose *et al*, 1990) and is neither glycosylated nor disulphide bonded (Aeschlimann & Paulsson, 1994). The complete amino acid sequences for tissue transglutaminase from guinea pig liver (Ikura *et al*, 1988), mouse macrophages and human endothelial cells (Gentile *et al*, 1991) have been deduced from the cDNA sequences. The guinea-pig liver and mouse tTG's show approximately an 80% homology to the human enzyme, with 49 of the 51 active site residues being identical (LeBlanc *et al*, 1999).

I-4.3.1 Regulation of tissue transglutaminase activity

Unlike the plasma, keratinocyte and epidermal transglutaminases, tTG does not require activation by proteolysis (Griffin & Verderio, 2000). Activation occurs by a conformational change induced by the binding of Ca^{2+} ions and the specificity of this cation binding appears to be high, as only Sr^{2+} can replace Ca^{2+} and activate the

enzyme, but then only at a 10-fold higher concentration. Two glutamine-rich regions of the tTG sequence, around amino acids 450 and 470, have been proposed for the calcium-binding sites (Ichinose *et al*, 1990).

However, the regulation of tissue transglutaminase activity involves more than the local calcium levels. Given that tTG is predominantly a cytosolic enzyme and can be activated at a Ca^{2+} concentration of around 10^{-6} M, its intracellular activity must be regulated by additional means.

This additional regulation may come from the binding of GTP and GDP to the enzyme (Smethurst & Griffin, 1996; Griffin & Verderio, 2000). GTP and GDP bind reversibly to tTG, and cause a conformational change in the enzyme which reduces its activation (Ichinose *et al*, 1990; Greenberg *et al*, 1991; Aeschlimann & Paulsson, 1994). Ca^{2+} ions inhibit this nucleotide binding and the local concentration of GTP/GDP and Ca^{2+} may therefore regulate tTG activity *in vivo* (Aeschlimann & Paulsson, 1994). The core domain of tTG may also hydrolyse GTP and ATP (Iismaa *et al*, 1997).

Also, tTG (in the absence of GTP/GDP binding) is an endogenous substrate of calpain. So whilst depleted GTP levels may raise tTG activity, they may also lead to tTG degradation by the subsequently elevated Ca^{2+} levels activating calpain (Zhang *et al*, 1998). This may be a further mechanism of regulating tissue transglutaminase activity by modulating its intra-cellular levels.

I-4.3.2 Regulation of tissue transglutaminase expression

The intracellular level of tTG is most often adjusted by changes in transcription (Aeschlimann & Thomazy, 2000). Different factors have been shown to affect tTG transcription in both cell lines and biological situations, but a direct link between a signalling pathway and tTG transcription has only established for a few of these cases.

These cases include retinoids and members of the TGF-β family (Aeschlimann & Paulsson, 1994). Tissue transglutaminase expression is enhanced by retinoic acid *in vivo* and *in vitro*, where induction occurs at physiological concentrations of retinoic

acid and leads to an intracellular accumulation of the enzyme (Moore *et al*, 1984; Nara *et al*, 1989; Piacentini *et al*, 1992).

I-4.3.3 Tissue transglutaminase and the extra-cellular matrix

The physiological function of tTG remains unclear and might be diverse in different tissues or biological events and pathological processes (Aeschlimann & Thomazy, 2000).

Although TG's are not conventionally secreted and the mechanism of their release from cells in unclear, the presence of tTG and other TG's in the ECM space is well documented (Aeschlimann *et al*, 1991; Aeschlimann *et al*, 1995; Martinez *et al*, 1994; Verderio *et al*, 1998).

A number of ECM proteins such as fibrinogen (Shainoff *et al*, 1991; Achyuthan *et al*, 1988), fibronectin (Fesus *et al*, 1986; Jones *et al*, 1997), vitronectin (Sane *et al*, 1988), nidogen/entactin (Aeschlimann & Paulsson, 1991), collagen type III propeptide (Bowness *et al*, 1987), collagen II, osteonectin (Aeschlimann *et al*, 1993; Aeschlimann *et al*, 1995) and osteopontin (Beninati *et al*, 1994) are substrates for tTG. Though not necessarily all *in vivo*. Cellular TG also demonstrates an affinity for the extra-cellular matrix and co-exists with fibronectin (Upchurch *et al*, 1987). This has led to tTG being proposed as a stabiliser of the ECM. The high calcium concentration and low nucleotide concentration of the extra-cellular environment provide the necessary conditions for activation of the enzyme.

An example of tissue stabilisation comes from the dermo-epidermal junction, where cross-linking of key structures by tTG provides stability at this tissue interface (Raghunuth *et al*, 1996). Cross-link is detected here and several components of the basement membrane are tTG substrates (nidogen/entactin, fibronectin, osteonectin, collagen VII) which occur as cross-linked complexes in tissues (Martinez *et al*, 1994; Raghunuth *et al*, 1996; Aeschlimann *et al*, 1995; Aeschlimann *et al*, 1991; Aeschlimann & Thomazy, 2000).

I-4.3.2 Tissue transglutaminase – role in cell-cell and cell-matrix interactions

Tissue transglutaminase may also have a role in cell-matrix and cell-cell interactions. Increased concentrations of the enzyme have been found at areas of cell-cell and cell-substratum contact (Gaudry *et al*, 1999) and tTG seems to be expressed at the surface of various cells such as fibroblasts, macrophages, hepatocytes and endothelial cells. Here the enzyme can contribute to both matrix assembly and cell-substrate interactions such as attachment and spreading (Aeschlimann & Thomazy, 2000; Martinez *et al*, 1994; Jones *et al*, 1997; Verderio *et al*, 1998).

This role in cell-matrix interaction may be mediated by the interaction with, and cross-linking of, cell-surface associated fibronectin (Martinez *et al*, 1994; Verderio *et al*, 1998; Jones *et al*, 1997).

Several studies have shown that tissue transglutaminase binds to fibronectin (Verderio *et al*, 1998; Gaudry *et al*, 1999; Jeong *et al*, 1995) and the cell-surface localisation of tTG is dependent on a fibronectin-binding site within the enzyme (Gaudry *et al*, 1999). Residues 1-7 of tTG seem especially important in its ability to bind calcium-independently to fibronectin (Jeong *et al*, 1995). In turn, fibronectin has tTG binding sites distinct from its cross-linking sites (Aeschlimann & Thomazy, 2000).

Tissue transglutaminase is responsible for the cross-linking of fibronectin during fibril assembly (Verderio *et al*, 1998). Cross-linked multimers of fibronectin (due to tTG expression) have been found at the basal surface of endothelial cell monolayers, where they may stabilise the ECM and anchor the cells to the basement membrane (Martinez *et al*, 1994).

Further evidence for the role of tTG in cell-matrix interaction comes from studies showing fibroblasts exhibiting reduced spreading and adhesion on a fibrin-fibronectin matrix, made with mutant fibronectin that lacks the major TG-cross linking site (Corbett *et al*, 1997).

I-4.3.2 Tissue transglutaminase – role in skeletal tissues

Matrix stabilisation by transglutaminases is part of the developmental program of the skeletal elements that form the structural framework of the vertebrate body (Aeschlimann & Thomazy, 2000).

Aeschlimann and co-workers (1993) suggested a correlation between the expression of tissue transglutaminase and the terminal differentiation of chondrocytes in endochondral bone formation and the calcification of maturing tracheal cartilage. The enzyme was expressed in the epiphyseal growth plate of rat long bones, firstly in the proliferation and maturation zone, where it accumulated intra-cellularly before being externalised during provisional calcification and prior to mineralisation. Studying homogenates of bovine cartilage, transglutaminase activity was also found in the growth plate tissue, but not in articular cartilage.

For tracheal cartilage (which mineralises but does not form bone), tTG was not found in 4-day old rat tissue, correlating with the resting chondrocytes in long bone not expressing the enzyme, but was found in adults in cells surrounding calcifying tissue at the borders of hydroxyapatite deposits. Similar results were also obtained for TG activity in tracheal rings.

Using antibodies raised against the γ-glutamyl-ε-lysine di-peptide, Aeschlimann *et al* (1995) then went on to show the presence of the cross-link (and proposed TG activity) in the mineralising matrix of the hypertrophic zone of growth plate cartilage and in the bone matrix of rat long bones. The pattern of cross-link distribution matched that found for tTG protein in cartilage, but the enzyme responsible for the cross-linking of bone matrix was thought to be an alternative transglutaminase. Cross-link was also detected in chondrocytes around mineral deposits in tracheal tissue in explant culture, found to increase with chondrocyte maturation and, is first detected around chondrocytes shortly before mineralisation. A proportion was also found in the inter-territorial and pericellular matrix in adult rat tissue. The phenotype of the chondrocytes around the tracheal mineral deposits resembles those of the hypertrophic zone of the long bone growth plate.

These results led to the proposal that transglutaminase expression, externalisation, activation and mineral formation were consecutive steps in the chondrocyte

maturation program in the post-natal development of tracheal cartilage and in endochondral ossification (Aeschlimann & Paulsson, 1996). The enzyme was proposed to be tissue transglutaminase in cartilage but an alternative member of the family in bone.

In contrast to the findings of Aeschlimann *et al* (1993), who found no TG activity in homogenates of bovine articular cartilage, rabbit articular chondrocytes in culture expressed two transglutaminase activities (Demignott *et al*, 1995). The relative proportion of these differed as a function of passage number, with the first being preferentially expressed at early passage, trypsin activated and membrane associated. The second was up regulated by retinoic acid, inactivated by trypsinisation of cell lysates and, was cytosolic.

Borge *et al* (1996) stated that the second of these two was tissue transglutaminase. They studied tTG expression induced in chondrocyte cultures, under conditions that stimulate cellular events that, in other cells, induce tTG expression.

Tissue transglutaminase was expressed in the chondrocytes at later passages, and only in cells undergoing de-differentiation. Retinoic acid treatment up-regulated expression regardless of passage number and expression was at higher levels when the cells were dividing. The enzyme was barely detectable in freshly isolated articular chondrocytes, which is consistent with the lack of tTG seen by immuno-staining in articular cartilage slices (Aeschlimann *et al*, 1993). The increased expression when cells were in the exponential phase also agrees with the previous findings, of no tTG activity in resting chondrocytes in the growth plate of long bones. As retinoic acid induces chondrocyte de-differentiation and tTG expression, it was suggested that the enzyme is involved in the de-differentiation of chondrocytes and a further proposal was a role in cell attachment, as there was a correlation between tTG expression and cell adhesion in this culture system.

Rosenthal and co-workers (1997) measured transglutaminase activity and tissue transglutaminase levels in old and young porcine articular cartilage, along with articular cartilage vesicles. They found a cytosolic TG activity that was seven fold

higher in the older cartilage (3-5 years) than the younger tissue (2-6 weeks). A membrane-associated activity was detected, but only in the older tissue, and articular cartilage vesicles also contained TG activity. The cytosolic TG activity was increased with $TGF\beta_1$ treatment and decreased by IGF-1, where as retinoic acid had no effect and the membrane-associated activity was unaffected by all.

Tissue transglutaminase protein was present in the cytosol of the older chondrocytes and in the vesicles, but barely detectable in the younger cells, and no tTG protein was found in the membrane fraction of the tissues.

These findings correlate some way with those of previous workers (Demignot *et al*, 1995; Borge *et al*, 1996), in that both found a cytosolic and a membrane TG activity, and that the former may be due to tissue transglutaminase.

The findings of the presence of TG activity and tTG protein in articular cartilage vesicles may be a further link between TG activity and the process of pathological biomineralisation that occurs in ageing cartilage. The vesicles act as sites of calcium pyrophosphate dihydrate crystal formation (CPPD) in cartilage, which are found in the development of destructive arthritis. The formation of the crystals is promoted by active $TGF\beta_1$ in articular cartilage and transglutaminase may play a role in either extra-cellular matrix alterations that favour vesicle formation, or in the activation of latent $TGF\beta_1$. Use of TG inhibitors blocked the media accumulation of inorganic phosphate, an essential precursor of crystal formation.

Further to this study, Rosenthal and co-workers (2000) measured the ability of old and young porcine articular chondrocytes to activate latent $TGF\beta_1$, in the presence and absence of TG inhibitors.

It was demonstrated that TG activates latent $TGF\beta_1$ in old chondrocytes, but its activation in young chondrocytes is independent of TG activity. Addition of TG inhibitors to old chondrocytes suppressed active $TGF\beta_1$ levels in the cell layer, but had no effect on young chondrocytes. This suppressive ability of the inhibitors was related to their ability to inhibit TG activity. Plasmin activity (an enzyme activator of latent TGFβ1) was unaffected by the TG inhibtors and did not alter between young

and old chondrocytes. The activation of latent TGFβ_1 shown by old chondrocytes may be in some part due to their increased levels of TG activity (Rosenthal *et al*, 1997).

However, this data suggests a novel pathologic function for TG in ageing articular cartilage and supports the theory of a role for elevated TG activity in age-related arthritis based on its participation in the activation of TGFβ_1 (which plays an important role in this degenerative process).

Attempts to characterise, using fluoroscein cadaverine (FC) labelling, the amine acceptor protein substrates accessible to active transglutaminase expressed by rabbit articular chondrocytes, were made by Lajemi *et al* (1998).

Following on from the previous findings which suggested the model of two different transglutaminases in differing proportions depending on passage number and retinoic acid treatment (Demignot *et al* 1995; Borge *et al* 1996) they found no labelling in primary chondrocyte cultures, but strong labelling at passage two. This was super-imposable over fibronectin staining and deemed to be extracellular, suggesting that these cells expressed at least type II transglutaminase (tTG).

The lack of extra-cellular labelling in primary cultures suggests that the FC was not accessible to acceptor sites in the matrix, or there was an absence of active TG. Addition of thrombin produced labelling which corresponded with that of fibronectin, as did addition of tTG, which suggests that articular chondrocytes in primary culture express an inactive, pro-enzyme form of TG, which needs proteolysis for activation and is partly extra-cellular. No FC labelling was found in the cytosol, though perhaps any tTG present is not in an environment that leads to its activation. Due to the tight regulation of its intracellular activity by calcium, GTP and GDP levels (see I-4.3.1).

When activated by the elevated Ca^{2+} levels in the extra-cellular matrix, tTG is thought to cross-link substrate proteins such as: osteonectin (Aeschlimann *et al*, 1995), collagen II (Aeschlimann *et al*, 1993), osteopontin (Beninati *et al*, 1994), fibronectin (Fesus *et al*, 1986), collagen V and collagen XI (Kleman *et al*, 1995; Aeschlimann & Paulsson, 1996).

The nature of a membrane associated pool of TG activity seen in chondrocytes is unknown and could be tTG or kTG. Factor XIII a sub-unit has also been isolated as an

up-regulated gene product upon differentiation of avian chondrocytes (Nurminskaya *et al*, 1998). But the extent to which these TG's contribute to cartilage maturation is not clear.

Transglutaminase cross-links are also abundant in bone matrix and osteoblasts have been shown to express TG activity (Aeschlimann *et al*, 1993). Tissue transglutaminase has also been demonstrated in human osteoblast-like cells (Heath *et al*, 2001). Cross-linked complexes of osteopontin have been isolated from bone matrix (for review see Aeschlimann & Paulsson, 1996) and tTG has been demonstrated to cross-link latent TGF-β binding protein 1 (LTBP-1) to bone extra-cellular matrix (Dallas *et al*, 1996). Tissue transglutaminase co-distributes with LTBP-1, suggesting that tTG expression may be important in controlling matrix storage of latent TGF-β_1 complexes, via the TG-catalysed cross-linking of LTBP-1 to fibronectin (Verderio *et al*, 1999).

I-4.3.4 Tissue transglutaminase – role in wound healing

Since tissue transglutaminase cross-links extra-cellular proteins and induces stabilisation of the extra-cellular matrix, it has been presented as a factor of wound repair.

An increase in tissue transglutaminase was shown during dermal wound healing after skin puncture (Bowness *et al*, 1988). Transglutaminase activities in all skin layers increased in the period 1-5 days after wounding. Similar experiments performed on embryonic human lung fibroblast cultures, showed an increase of bound tissue transglutaminase to the extra-cellular matrices at the wounding sites for many hours after damage (Upchurch *et al*, 1991). This suggests a binding of the enzyme leaking out from injured cells, having a repair action and helping in the wound healing process. Raghunath and co-workers (1996) reported similar observations made on human skin where tissue transglutaminase seemed to be implicated in the regeneration process of grafts. They concluded that the role of tissue transglutaminase in the healing process of the grafts was a stabilisation of the anchoring fibres of the dermo-

epidermal junction since collagen VII was identified as a tissue transglutaminase substrate.

Tissue transglutaminase is present in vascular walls and contributes, along with Factor XIII, to hemostasis and wound healing by stabilisation of fibrin clot and endothelial extra-cellular matrix following injury (Greenberg *et al*, 1987). Many plasma proteins that contribute to platelet-fibrin-endothelium interactions and to the wound healing process, are tTG substrates (Aeschlimann & Thomazy, 2000; Greenberg *et al*, 1987). A variety of pathological conditions in which tTG appears to participate are associated with its role in wound healing.

I-4.3.5 Tissue transglutaminase – involvement in pathological disorders

Increased TG activity and tTG specific cross-linked products are found in the different stages leading to cataract formation (Lorand & Conrad, 1984) and in atherosclerotic plaque formation (Bowness *et al*, 1994). Tissue transglutaminase is also up regulated in rheumatoid arthritis, but not osteoarthritis (Weinberg *et al*, 1991). Increased transglutaminase related cross-linking is found in experimental renal scarring, where it stabilises the extra-cellular matrix and its increased expression is likely to contribute to the expansion of the extra-cellular matrix that is seen in renal fibrosis (Johnson *et al*, 1999). Tissue transglutaminase has also been implicated in neuro-degenerative diseases, such as Huntingdon's disease (Igarishi *et al*, 1998) and Alzheimers (Johnson *et al*, 1997), and Celiac disease (Lock *et al*, 1999).

The association of increased synthesis of tTG and the accumulation of its extra products with inflammation, wound healing and fibrotic processes suggests that tTG induction is part of a cellular stress response. This stress response may be misplaced (as in the neuro-degenerative disorders and cataract formation), exaggerated (fibrosis and atherosclerosis) or provoke auto-immunity (celiac disease) leading to pathological disorders (Aeschlimann & Thomazy, 2000).

I-4.3.6 Tissue transglutaminase - GTPase activity and cell signalling

The capacity of tissue transglutaminase to bind and hydrolysis GTP is a feature not associated with the other TG's, and an additional function for tTG has been proposed as a G-protein in hormone receptor signalling (Im *et al*, 1997).

I-4.3.7 Tissue transglutaminase and cell death

Tissue transglutaminase has been postulated to play a role in both of the two classically defined methods of cell death, necrosis and apoptosis.

In apoptotic cell death, transglutaminase was shown to be over-expressed as the cell died and to cross-link proteins within the dying cell (Knight *et al*, 1991). The cross-linking of intracellular components and apoptotic bodies by tTG was thought to be stabilising the apoptotic cells before their clearance by phagocytes (to prevent leakage of cellular components into the extra-cellular matrix). But the occurrence of apoptosis, and tTG expression, don't always completely overlap. Tissue transglutaminase expression is usually induced in the earlier stages of apoptosis, suggesting an additional role to the cross-linking function. But results are contradictory as to the role and importance of tTG in the apoptotic pathway (Griffin & Verderio, 2000).

Current data suggests that up regulation of tTG is not a specific hallmark of apoptosis but that the enzyme can be recruited during cell death (both necrosis and apoptosis). Where it may function as an effector molecule of apoptosis in distinct biological situations, perhaps cross-linking structural proteins to maintain cell integrity and prevent an inflammatory response (Aeschlimann & Thomazy, 2000)

Wide ranges of responses to cellular damage do not meet the stereotyped criteria for apoptosis or necrosis, suggesting that there may be alternative, unclassified modes of cell death. Tissue transglutaminase may induce an alternative form of cell death, consequent to a sustained intracellular calcium elevation, which is characterised by cell stabilisation by tTG cross-links following injury (Griffin & Verderio, 2000).

I-4.4 Transglutaminases - Biomedical applications

Transglutaminases have been suggested for a variety of biomedical applications in the field of wound healing and tissue repair. Since they create covalent cross-links between extra-cellular matrix proteins, they are prime candidates for stabilising tissues and hence many of these applications relate to tissue bonding.

A wound healing composition comprising a calcium-independent microbial transglutaminase was proposed by Kitahara *et al* (1996), either to be used on its own or with a dressing, that would allow wound protection and coating. Mawatari & Usei (1995) also made claim of a living tissue adhesive, based on either guinea-pig liver tissue transglutaminase or the microbial enzyme (thought preferable for easier production).

The aim of this research was to investigate transglutaminases as tissue bonding agents, specifically tissue transglutaminase.

The most significant work to date in this regard was undertaken by Jurgensen and co-workers (1997), who used an *in vitro* model to test the capacity of tTG to increase the adhesive strength at a cartilage-cartilage interface (in response to shear stress). Their findings will be discussed in more detail later, with respect to those achieved in this study, but in general they found adhesive strength to increase linearly with tTG concentration. Bond strength was also enhanced by an increased duration of incubation, but was not influenced by the level of humidity during incubation. When compared to the performance of a commercial fibrin sealant using the same model, the results for tTG matched, and in some instances exceeded, the bonding strength of the commercial adhesive.

Applications of transglutaminase are not limited to use of the enzyme alone. A transglutaminase-based adhesive may be used in conjunction with TG cross-linkable polypeptides in homo- and co-polymer formulations that can be cross-linked by the TG to add stability to the biomaterial, form matrices and enable the biomaterial to adhere to tissue surfaces (Labroo *et al*, 1995). Tissue transglutaminase may thus be used in conjunction with existing materials to facilitate their use and alter/improve their properties

Biomedical applications for transglutaminases, not related to tissue adhesion, include Factor XIII treatment for FXIII deficient patients with bleeding episodes or inability to carry pregnancy to term, and for patients with chronic wound conditions such as leg ulcers (Aeschlimann & Thomazy, 2000).

I-5 Aims

There are many situations within the different surgical specialities where the application of a biological-glue would be useful. The aim of the following thesis was to investigate the potential of transglutaminases, specifically tissue transglutaminase, to act as tissue bonding agents. An *in vitro* model was developed to measure the capacity for transglutaminase to increase the bond strength at a cartilage-cartilage interface (in response to tensile stress). The intention being for the primary application of the tTG-based biological-adhesive to be in the field of orthopaedics.

As previously outlined (see I-3.5), current cartilage repair treatments are often undermined by the failure of repair materials to sufficiently adhere to the surrounding host tissue. The study of the bonding capacity of tissue transglutaminase would include attempts to improve bonding by altering both the adhesive composition and the tissue surfaces to be bonded, along with investigating the bonding of biomaterials to cartilage surfaces. A further *in vitro* model would also be used to investigate the viability of chondrocytes at wound edges, in articular cartilage-explant culture. The aim was to reduce the loss of viability seen at the wound edges and measure the effects that adhesive application may have on cells at the wound edge of the recipient tissue.

Chapter II : Materials and General Methods

Chapter II : Materials and general methods

II-1 Materials

All chemicals were obtained from SIGMA (Poole, UK) unless stated otherwise.

Lloyds 6000R testing machine from Lloyds Instruments Ltd, Fareham, UK.

[1,4-^{14}C]-putrescine from Amersham Life Sciences, Little Chalfont, UK.

Whatman 3M filter papers from Whatman international Ltd, Maidstone, UK.

Optiphase High Safe Liquid Scintillant and Packard Liquid Scintillator from Packard Instrument Company, Meriden, USA.

Lowry protein assay commercial kit from Bio-Rad, Hemel Hemptsead, UK.

ELISA plate reader Titertek Multiscan MCC/340 from Labsystems, Helsinki, Finland.

SDS-PAGE gel apparatus from ATTO-Corporation, Japan.

Tissue culture plastic ware ordered from Corning, New York, USA.

CO_2 incubator (Model IG150) and sterile cabinet (Gelaire BSB 4A) from Jouan, Tring, UK.

Beriplast® and Fibrogammin® from Centeon, now Aventis-Behring, Haywards Heath, UK.

Leica TCS-NT confocal laser scanning microscope from Leica, Milton Keynes, UK.

Superfrost Plus Gold slides and OCT tissue embedding medium from BDH Lab Supplies, Poole, UK.

Hydrophobic pen and Vectorshield fluorescent mounting medium from Vector Labs Inc, Burlinggame, USA.

Fluorescein cadaverine, Texas-Red cadaverine and Live/Dead® Viability/cytotoxicity Kit from Molecular Probes, Oregon, USA.

Z-VAD FMK caspase inhibitor from Promega, Madison, USA.

Biopsy punches from Stiefel, High Wycombe, UK.

Activa EB and KHM2 microbial transglutaminase preparations from Ajinomoto Co, (Japan) courtesy of Forum Holdings, Redhill, UK.

II-2 Methods in measuring bond strength

II-2.1 Preparation of tissue samples

Cartilage was taken from the sternum end of adult porcine ribs, obtained from local butchers. The ribs were stored at –20°C until collection, after which the cartilage was removed and cleaned of surrounding tissue. The cylindrical samples were cut to length (4-5cm), wrapped in foil to prevent drying and stored at –20°C until use. Prior to testing, samples were thawed at room temperature in foil to prevent de-hydration.

II-2.2 Measurement of bond strength

An *in vitro* model was developed to measure the capacity for tissue transglutaminase, and other solutions, to increase the bond strength at a cartilage-cartilage interface in response to tensile stress.

Cylindrical cartilage samples were thawed at room temperature from storage at -20°C.

Samples were clamped between two specially designed, stainless steel holding devices that held the cartilage via 12 trapezium-ended screws. These were arranged in an Y-shaped orientation, with 6 screws per section.

Samples were then cut perpendicular to the intended direction of separation upon testing, using a fresh post-mortem blade. This provided a horizontal cartilage-cartilage interface between the two clamps, upon which to test the adhesive nature of applied solutions.

Solutions to be tested were applied to the cut surfaces, which were then re-opposed creating a 'butt-jointed' sample. The whole assembly was placed in a specially designed collar and clips that ensured the cut surfaces were re-opposed in the correct orientation and held immovable during incubation. The apparatus was then placed at 37°C.

A 2 KG weight was applied vertically to the tissue sample in order to keep the cut surfaces opposed during incubation. 2 KG allowed sufficient contact between surfaces for bonding, but did not lead to samples slipping within the holding devices.

Incubation times and humidity were varied to test their influence on the adhesive strength achieved at the interface.

For measurement of bond strength at the cartilage-cartilage interface, the assembly was loaded into a Lloyds 6000R testing machine fitted with a 20N capacity load cell, and the collar and clips subsequently removed.

The machine separated the cartilage sample in a vertical fashion at a definable speed (5mm/min) and measured the force (N) required to separate the sample along the cartilage-cartilage interface. This maximum force at the point of failure was taken as a measure of the bond strength of the solutions applied to that interface.

Bond strength is expressed in units of megapascalls (MPa) which are equivalent to N/mm^2 (calculated after measuring the surface area of the interface tested).

At least four specimens were evaluated for each parameter that was tested, and results are given as the mean and standard error.

A schematic diagram of the procedure is shown in figure II-1 and photographs of the clamp assembly and testing machine are shown in figure II-2

Figure II-1 Schematic diagram of the assembly of tissue samples for measuring bond strength in response to tensile stress at cartilage-cartilage interfaces

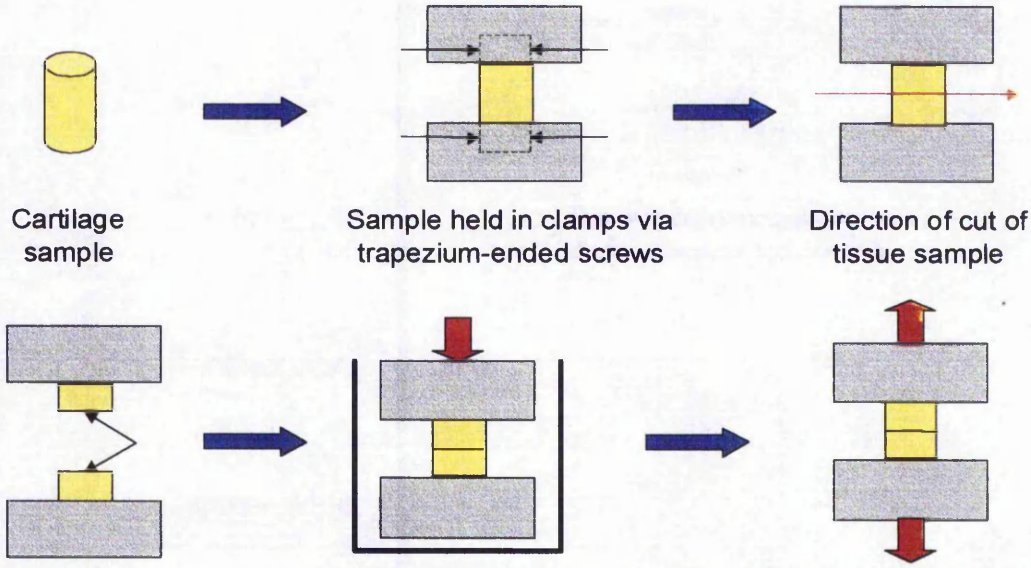
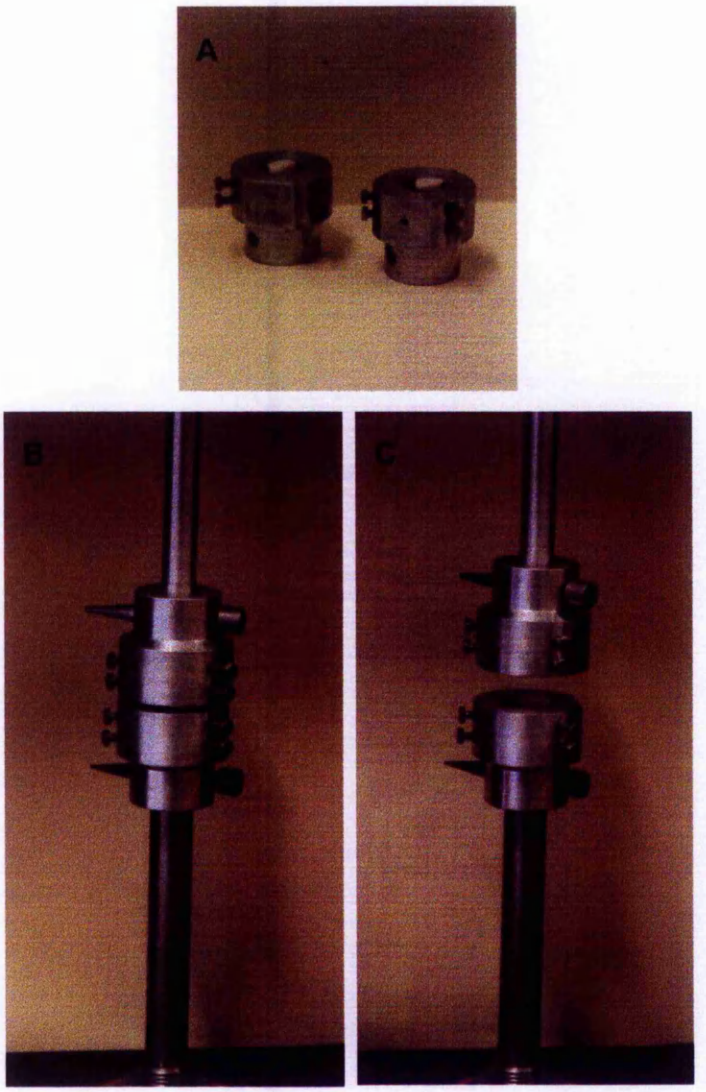


Figure II-2 *In vitro* system for measuring bond strength at cartilage-cartilage interfaces



A) Tissue sample in clamps with cut cartilage surfaces ready for adhesive application

B) Tissue sample within clamps, loaded into testing machine for separation of bonded cartilage-cartilage interface

C) Tissue sample within clamps, following separation of bonded cartilage-cartilage interface

II-3 Transglutaminase activity assay: [^{14}C]-putrescine incorporation into N-N'dimethylcasein

Transglutaminase activity was measured as described previously (Knight *et al*, 1990; Verderio *et al*, 1998) using the TG-catalysed reaction of [^{14}C]-putrescine incorporation into N-N'dimethylcasein, which forms a TCA-insoluble material.

The reaction was carried out at 37°C in 1.5ml centrifuge tubes after addition of the following:

50mM Tris.Cl pH 7.3	10µl
38.5mM DTT (in 50mM Tris.Cl pH 7.3)	10µl
50mM CaCl (in 50mM Tris.Cl pH 7.3)	5µl
12mM [1,4-^{14}C]-putrescine (specific activity 3.97µCi/µmol)	10µl
25mg/ml N,N'-dimethylcasein (in 50mM Tris.Cl pH 7.3)	20 µl
Sample	45 µl

Whatman 3M filter paper squares (100mm^2) were used to sample the reaction.

[1,4-^{14}C]-putrescine stock was made by the addition of 50µl of 243mM cold putrescine (in 1.05M Tris.Cl pH 7.4) to 1ml of [1,4-^{14}C]-putrescine.

At 10 and 20 minutes following initiation of the reaction (via addition of sample) 10µl aliquots were removed in triplicate from the reaction tubes and spotted onto appropriately labelled individual filter paper squares. These were then placed in ice-cold 10% (v/v) TCA for 20 minutes before the following washes were performed:

1 x 10% (v/v) ice cold TCA for 10 minutes

3 x 5% (v/v) ice cold TCA for 5 minutes

1 x 1:1 acetone:ethanol for 5 minutes

1 x acetone for 5 minutes

As a control for each sample, the reaction was also performed using 5µl of 100mM EDTA (in 50mM Tris.Cl pH 7.3) in place of the calcium chloride solution.

To determine the efficiency of counting, a further triplicate of 10µl aliquots of reaction mixture were removed and spotted onto filter paper squares. These were not subjected to any subsequent washing in order to measure the total counts in the assay.

Finally, after drying of the filter papers over-night at room temperature, they were placed individually into 2ml of Optiphase High Safe scintillant and counted for 5 minutes per sample in a Packard Liquid Scintillator. Specific transglutaminase activity is expressed in nmol of putrescine incorporated per hour (unit of activity) per mg of protein (U/mg).

II-4 Determination of protein content

The total protein content of samples was determined using a commercial kit (Bio-Rad) that is based on the Lowry method. The kit was used according to manufacturers' literature, which comprised, in brief, the following.

5µl of sample was added per well to a 96-well plate, with three repeats per sample. 25µl of 'reagent A' and 250µl of 'reagent B' were added per well, followed by incubation of the plate for 10 minutes at room temperature. Absorbances of each well were measured at 670nm using an Elisa plate reader (Titertek Multiscan MCC/340) with bovine albumin solutions used as standards of known protein concentration.

II-5 Polyacrylamide gel electrophoresis (PAGE)

The polyacrylamide gel is obtained after polymerisation of acrylamide with N-N'-methylene bis-acrylamide. A reaction initialised by ammonium persulphate and catalysed by TEMED. The method used is a modification of the Laemmli method (Laemmli, 1970) for use in vertical slab gels and its recipes are according to those found in 'Current protocols in molecular biology, Volume 2' (Ausubel *et al*, 1991). Discontinuous gels with a 3% (w/v) stacking gel and 10% (w/v) resolving gel were prepared according to the following recipes.

Stacking gel

30% acrylamide/0.8% bisacrylamide	0.65ml
4X TrisCl/SDS pH 6.8	1.25ml
dH_20	3.05ml
10% ammonium persulphate	0.025ml
TEMED	0.005ml

Resolving gel (10% w/v acrylamide content)

30% acrylamide/0.8% bisacrylamide	5.00ml
4X TrisCl/SDS pH 8.8	3.75ml
dH_20	6.25ml
10% ammonium persulphate	0.05ml
TEMED	0.01ml

Tris/SDS stock solution, pH 6.8 - 0.25M Tris base, 0.2% (w/v) SDS

Tris/SDS stock solution, pH 8.8 - 0.75M Tris base, 0.2% (w/v) SDS

PAGE was performed using ATTO-corporation gel apparatus and ran at 150V until the Bromophenol blue tracking dye in the sample buffer had reached the bottom of the resolving gel. Protein bands were then visualised using Co-massie blue stain.

Running buffer	2X Laemmli sample buffer	Co-massie blue stain
0.025M Tris base	50mM Tris.Cl pH 6.8	0.1% Comassie blue
0.192M Glycine	2% (w/v) SDS	40% (v/v) Methanol
0.1% (w/v) SDS	100mM DTT	10% (v/v) Acetic acid
pH 8.5	0.1% (w/v) Bromophenol blue	
	10% (v/v) glycerol	

Chapter III : Bond strength of tissue transglutaminase at cartilage-cartilage interfaces

Chapter III : Bond strength of tissue transglutaminase at cartilage-cartilage interfaces

III-1 Introduction

As cartilage is subjected to great stress *in vivo*, an enzyme that helps stabilise the tissue could play a key role in maintaining cartilage integrity during repair.

The capacity of tissue transglutaminase to act as a biological-glue at a cartilage-cartilage interface was assessed with the use of a novel *in vitro* test method for quantifying bond strength (see II-2). This system measures bond strength in response to tensile stress.

The capacity of tissue transglutaminase to act as a cartilage adhesive in response to shear stress has previously been investigated by Jurgensen *et al* (1997, see also I-3.4).

In this previous study, a cartilage cylinder was cut transversely to provide two freshly cut surfaces. Tissue transglutaminase was then applied to one surface and a calcium chloride activating solution applied to the other. The two surfaces were opposed, one on top of the other, and a weight applied to the upper half of the cylinder for a period of defined time, temperature and humidity. The adhesive force at the interface was measured by applying a transverse force to the upper half of the cylinder. The changes in the adhesive strength produced at the tissue interface by tTG were investigated in response to variation of incubation conditions, and in comparison with a commercial fibrin sealant. Tissue transglutaminase was shown to be capable of bonding a cartilage-cartilage interface with equivalent, and in some cases better, strength than that of the commercial fibrin sealant.

Cartilage is placed under a variety of tensile and shear stresses during loading and joint motion and so if tissue transglutaminase is to serve as a cartilage adhesive, then its bonding properties need to be investigated in response to tensile as well as shear stress. Work presented here uses the studies of Jurgensen and co-workers (1997) as a basis and comparison point for further investigation.

The decision to base an adhesive on tissue transglutaminase is founded on several of the enzymes' properties.

Firstly, as cross-linkers of extra-cellular matrix proteins, transglutaminases are prime candidates for stabilising tissues during wound healing (Raghunuth *et al*, 1996).

In addition, tTG has a broader specificity for substrates than Factor XIII (the transglutaminase utilised in fibrin sealants see I-2.3) and is therefore more likely to produce stronger adhesion on a wider variety of tissues. tTG can act on a less restricted group of peptide bound glutamines than Factor XIII and produce more cross-linking at a tissue interface.

Tissue transglutaminase based adhesives may also not require a protein additive, as there is no proteolytic activation of tTG. Calcium ions can be used as an activator to give easier reproducible results and eliminate the need for the blood-product thrombin in the preparation (a fact considered a disadvantage of fibrin sealants, see I-2.3.4).

Finally, when applied to cartilage, the sealant may be mimicking a naturally occurring process (TG-catalysed cross-linking within the tissue, see I-3.3.2) and so the enzyme and its recombinant products would be expected to be non-toxic (Jurgenson *et al*, 1997; Aeschlimann *et al*, 1996).

Bonding of a cartilage-cartilage interface by tissue transglutaminase was studied here in response to duration of incubation, incubation humidity, enzyme concentration and pre-treatment of tissue surfaces. The bond strength of a commercial fibrin sealant, Beriplast®, was also studied in response to many of these conditions, giving a direct comparison for the performance of the tTG adhesive. This type of 'internal control' is lacking in many published adhesive investigations, which often fail to compare preparations under controlled conditions (see I-2.3.2).

III-2 Methods

III-2.1 Tissue transglutaminase

Guinea-pig liver tTG was re-constituted in filter-sterilised 50mM Tris.Cl buffer (pH 7.3) and stored in aliquots at -20°C until use. Each batch of tTG was assessed as a quality control measure, for activity via radio-labelled putrescine incorporation into N,N'-dimethylcaesin (see II-3) and for its bond strength at 4mg/ml under standard conditions (see III-2.1.1). Within each variable investigated, bond strength comparisons were made using the same batch of tTG unless otherwise stated.

III-2.1.1 Measurement of tissue transglutaminase bond strength

Measurement of tTG bond strength was performed using the novel method described (II-2). tTG solution (5µl) was applied to one cut cartilage surface and 5µl of a 'Tris activating buffer' to the other (in order to prevent premature activation of the enzyme). 'Tris activating buffer' (TAB) was a 0.1M-CaCl solution in 0.01M-Tris.Cl buffer containing 0.3M-NaCl (pH 7.3) (Jurgensen *et al*, 1997). For control comparisons, 50mM Tris.Cl buffer (pH 7.3) was used in place of tTG solution.

The volume of applied solutions was kept constant unless stated otherwise, and incubation conditions were varied to judge their influence on bond strength. Standard incubation conditions were defined as 20 min at 37°C and 20% humidity.

The effect on the bond strength of tTG preparations was also investigated with respect to the inclusion of various additional proteins. These were re-constituted in 50mM Tris.Cl buffer (pH 7.3) and combined with tTG solution immediately prior to application to the interface. Concentrations given (for tTG also) are with respect to the 5µl of solution applied. 5µl of TAB was also applied as standard.

III-2.2 Commercial fibrin sealant – Beriplast®

Tissue transglutaminase bond strength was compared to that of Beriplast® (Centeon, UK), a commercially available fibrin sealant that was handled and stored according to manufacturers' literature.

The product consists of four vials, one and two being used to make a fibrinogen solution, three and four to make a thrombin solution.

A 1ml Beriplast® combi-set contains:

VIAL 1 Human plasma proteins; Fibrinogen 65-115 mg, Factor XIII 40-80 U, Albumin 5-15 mg, NaCl, sodium citrate-dihydrate, L-isoleucine, sodium-L-glutamate-monohydrate, L-arginine hydrochloride.

VIAL 2 1000 KIU bovine lung aprotonin (0.56PEU), NaCl, water for injections.

VIAL 3 Human thrombin 400-600 IU, NaCl, sodium citrate-dihydrate.

VIAL 4 Calcium chloride-dihydrate (40mmol/l), water for injections.

III-2.2.1 Measurement of Beriplast® bond strength

Measurement of Beriplast® bond strength was performed using the novel method described (see II-2). Beriplast® fibrinogen solution was applied to one cut cartilage surface and then immediately over-layed with thrombin solution. The two tissue surfaces were then re-opposed and the assembly incubated as outlined previously.

III-2.2.2 SDS-PAGE analysis of Beriplast® activity

To demonstrate the activity of Beriplast® adhesive (intrinsically the polymerisation of fibrinogen sub-units during Factor XIII catalysed cross-linking), SDS-PAGE was performed using 10% acrylamide content resolving gels (see II-5).

A 1ml Beriplast® combi-set was re-constituted according to manufacturers' instructions and used to make two samples:

1. Beriplast® Fibrinogen solution alone
2. Beriplast® Fibrinogen solution, plus an equal volume of Beriplast® Thrombin/CaCl solution.

Both samples were incubated at 37°C for 20 minutes, before addition of 40µl of 9M Urea, 5% SDS, 5% β-mercaptoethanol and subsequent incubation at 70°C for 30 minutes. 40 µl of 2X Laemmli sample buffer (see II-5) was added to each sample, which were then incubated at 100°C for 25 minutes before being placed on ice. Samples were diluted a further 1:10 in sample buffer and 5µl of these loaded per lane

of the gel. Broad-range SDS-PAGE Mr markers (5µl, SIGMA) were ran at the same time.

III-2.3 Pre-treatment of cartilage-cartilage interfaces

Before application of tTG, various pre-treatments were performed on the exposed surfaces of the cartilage-cartilage interface, to see their effect on the bond strength achieved by tTG at the interface.

III-2.3.1 Physical etching

Both cut cartilage surfaces were scored with a surgical scalpel in a grid-pattern, with score lines approximately 2mm apart and 2-3mm deep.

III-2.3.2 Drying

Cut cartilage surfaces were exposed to a dual tungsten filament heat lamp for 5 minutes at a distance of 10 cm.

III-2.3.3 Exposure to UV irradiation

Both cut cartilage surfaces were exposed to an UV emitting lamp for 20 minutes, at a distance of ~ 10 cm. The lamp emitted UV radiation at 290-320nm wavelength, corresponding to approximately 99% production of UVA and 1% of UVB. Average energy emitted from the lamp for each exposure was 9007 KJ.

III-2.3.4 Enzymatic digestion of glycosaminoglycans (GAG's) and/or collagen from the tissue surfaces

Both cut cartilage surfaces were pre-treated to digest GAG's from the tissue surface. 20µl of enzyme solution (at different concentrations) was applied per surface, which were then incubated at 37°C, 20% humidity for 5 or 15 minutes. This was followed by

thorough rinsing of the surfaces with phosphate buffered saline and careful drying with filter paper, before application of tTG and TAB solutions as standard.

Enzyme solutions: Chondroitinase AC lyase (EC 4.2.2.5 from *Flavobacterium heparinum*), Chondroitinase ABC lyase (EC 4.2.2.4), Hyaluronidase (EC 3.2.1.35 Type 1-S from bovine testes), Heparinase I (EC 4.2.2.7 from *Flavobacterium heparinum*). All re-constituted in 50mM Tris.Cl buffer (pH 7.3).

Both cut cartilage surfaces were also pre-treated to digest collagen at the tissue surface. This occurred as for the GAG-digestion, with collagenase solution (from *Clostridium histolyticum*, EC 3.4.24.3, re-constituted in 50mM Tris.Cl buffer (pH 7.3)) in place of GAG-digesting enzyme.

II-2.4 Statistics

The data relating to bond strength was compared by two-way analysis using the students' t-test (assuming unequal variance). Significant differences ($p<0.05$) are labelled as *.

III-3 Results

III-3.1 Bond strength of tissue transglutaminase at cartilage-cartilage interfaces

III-3.1.1 Influence of tTG concentration

The bond strength of tTG at a cartilage-cartilage interface exhibited an approximately linear dependence on enzyme concentration (range 2-8 mg/ml) as shown in Figure III-1. The column designated '0mg/ml' represents the bond strength achieved with buffer alone (control solution) and is less than the strengths achieved with tTG preparations. A statistically significant ($p<0.05$) increase in bond strength (compared to control solution) was shown by the 8mg/ml tTG preparation.

III-3.1.2 Influence of tTG inhibitor

In the presence of the transglutaminase inhibitor EDTA (5mM), the bond strength of tTG at a cartilage-cartilage interface reduces to a level equivalent to that shown by the buffer control solution (Figure III-2). Under standard incubation conditions, tTG (4mg/ml) showed a bond strength of 0.047 ± 0.010 MPa, compared to 0.023 ± 0.002 when used in the presence of EDTA (5mM). The control solution showed a bond strength of 0.025 ± 0.003 MPa.

This indicates that tTG is playing an active role in the bonding process at the tissue interface.

Figure III-1 Bond strength achieved at a cartilage-cartilage interface with use of tissue transglutaminase (tTG) at varying concentrations, under standard incubation conditions

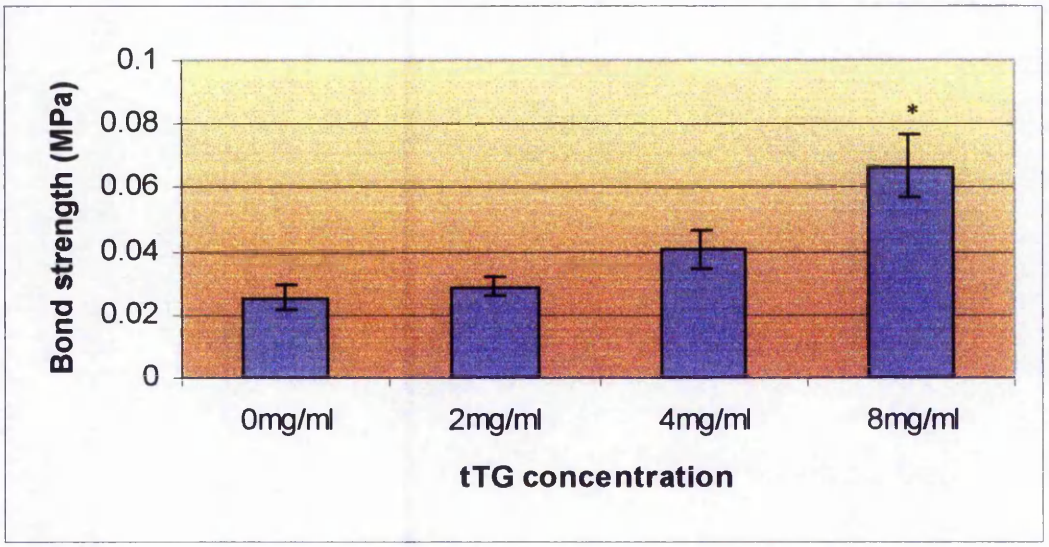


Figure III-1.

Bond strength of tTG (at varying concentration, 3447 nmol/hr/mg specific activity) after incubation for 20min at 37°c, 20% humidity (for method, see II-2.1.1). Figures show mean bond strength ± standard error (n=4). Statistically significant data (versus '0mg/ml' control) are marked as * ($p<0.05$).

Figure III-2 Bond strength achieved at a cartilage-cartilage interface with use of tTG, with and without the presence EDTA, and buffer control, under standard incubation conditions

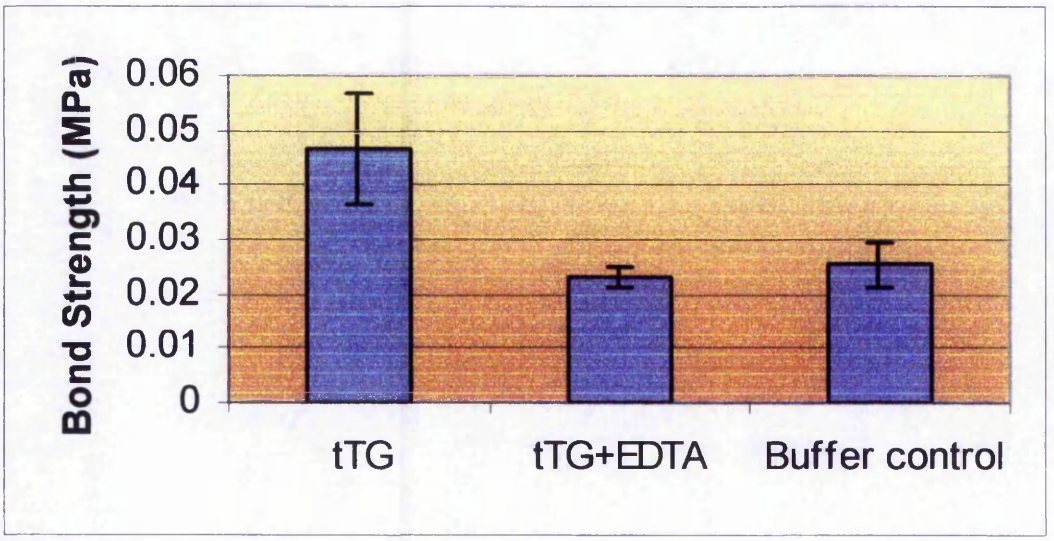


Figure III-2.

Bond strength of tTG (4mg/ml, 1622 nmol/hr/mg specific activity, with and without EDTA (5mM)), and buffer control after incubation for 20min at 37°c, 20% humidity (for method, see III-2.1.1). Figures show mean bond strength ± standard error (n=4).

III-3.1.3 Influence of varying incubation times

The bond strength shown by tTG at a cartilage-cartilage interface also increased as a function of time, a phenomenon that was observed for the commercial fibrin sealant Beriplast® as well (Figure III-3).

Using two concentrations of tTG preparation (4 & 8 mg/ml, taken from two different batches of tTG) it was found that tTG bond strength approached a maximum plateau value after incubations of forty minutes. This value was 0.045 ± 0.004 MPa for the 4mg/ml preparation and 0.094 ± 0.012 MPa for the 8mg/ml solution. Over all three incubation times tested (10, 20 & 40 minutes), tTG showed comparable bond strengths to those of the commercial fibrin sealant when used at a concentration of 8mg/ml. Although a small batch to batch variation in activity and bonding strength was observed for the commercial tTG.

Statistically significant ($p<0.05$) increases in bond strength were shown by tTG (8mg/ml) and Beriplast® after 20 minutes incubation compared to 10 minutes.

The bond strength of the buffer control solution did not exhibit a similar relation to incubation time, which suggests that the increased bond strengths achieved by tTG after longer incubation periods were not simply due to the sample drying out and becoming 'tacky' along the tissue interface.

III-3.1.4 Influence of varying incubation humidity

The bond strength of tTG preparations at a cartilage-cartilage interface was maintained at incubation conditions ranging from 20-100% humidity (Figure III-4). Whereas, Beriplast® exhibited a statistically significantly ($p<0.05$) reduction in bond strength when incubation humidity reached 90-100% (compared to incubation at 20-30% humidity). The buffer control solution showed no change in bond strength over the humidity range.

tTG (4mg/ml) showed a bond strength of 0.041 +- 0.006 MPa when incubated for 20 minutes at 20-30% humidity, 0.040 +- 0.001 MPa when used at 60-70% humidity and 0.039 +- 0.005 MPa at 90-100% humidity. This indicates that tTG should maintain its bond strength in fluid-filled atmospheres, which is desirable for a tissue adhesive intended for application in the wet environments found *in vivo*.

Figure III-3 Bond strength achieved at a cartilage-cartilage interface with use of tTG, Beriplast® or buffer control, measured as a function of duration of incubation

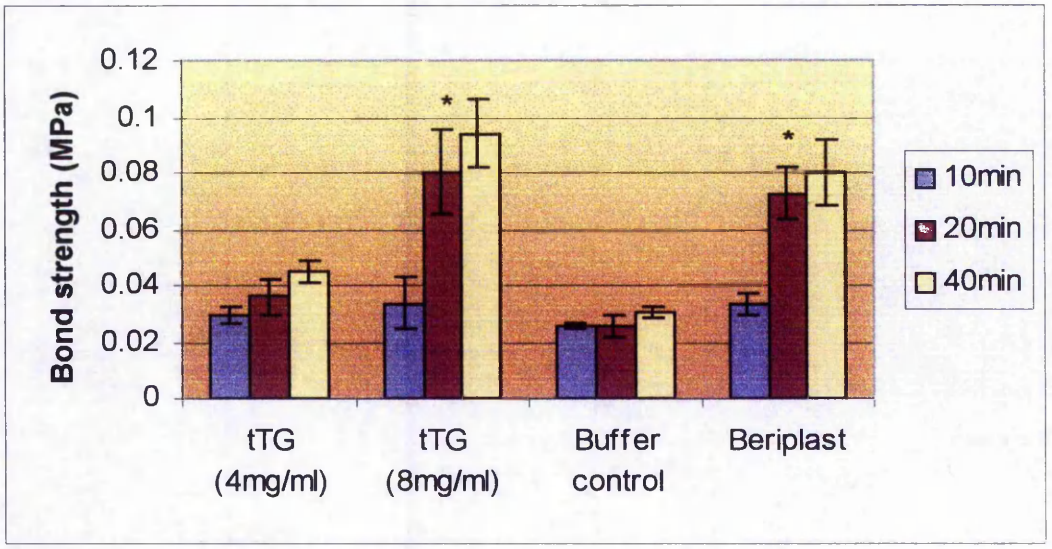


Figure III-3.

Bond strength of tTG (4 & 8 mg/ml, 3447 & 4172 nmol/hr/mg specific activity respectively), Beriplast and buffer control, after incubation for 10, 20 & 40 min at 37°c, 20% humidity (for method, see III-2.1.1 and III-2.2.1). Figures show mean bond strength ± standard error (n=4). Statistically significant data (compared to previous incubation within each group i.e. 20min compared to 10min, 40min compared to 20min) are marked as * ($p<0.05$).

Figure III-4 Influence of the % humidity during incubation on the bond strength achieved at a cartilage-cartilage interface with use of tTG, Beriplast® and buffer control

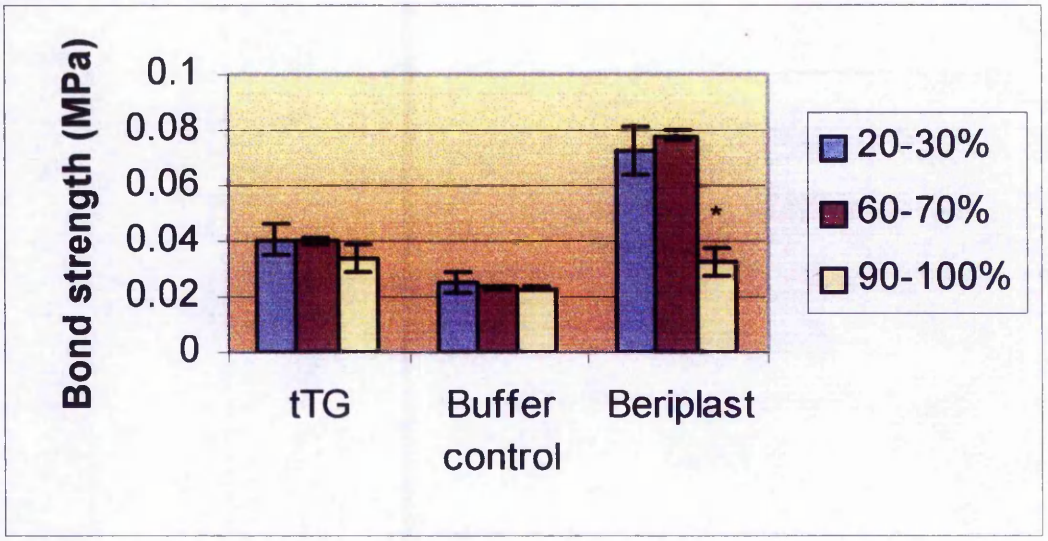


Figure III-4.

Bond strength of tTG (4mg/ml, 3447 nmol/hr/mg specific activity), Beriplast and buffer control, after incubation for 20min at 37°c, 20-30% humidity, 60-70% humidity and 90-100% humidity (for method, see III-2.1.1 and III-2.2.1). Figures show mean bond strength ± standard error (n=4). Statistically significant data (compared to incubation at 20-30% humidity within each group) are marked as * ($p<0.05$).

III-3.2 SDS-PAGE analysis of Beriplast ® polymerisation

Beriplast® manufacturers' literature states that 'tissues requiring adhesion should be fixed in place for several minutes until provisional adhesion is achieved'. This provisional adhesion (and the structural rigidity of fibrin sealant) is due to the action of thrombin and activated Factor XIII cross-linking the structural protein, fibrinogen.

Fibrinogen is a 340 Kda plasma protein that exists in dimer form, with each identical unit being made up of three polypeptide chains termed α, β and γ. After mixing of the two component solutions of Beriplast® (fibrinogen solution and thrombin/CaCl solution), the fibrinogen contained therein is transformed to fibrin monomers by the action of thrombin. The subsequent covalent cross-linking introduced into the fibrin sealant by Factor XIIIa (from thrombin activation of Factor XIII) leads to the formation of both γ-γ chain dimers and α chain polymers (this second reaction taking up to 24 hours for completion). This transamination cross-linking of fibrin-monomers produces covalent peptide bridges between fibrin chains, forming a high Mr polymer that increases the gel's rigidity of sealing (Sierra, 1993). The formation of these cross-linked complexes from the sub-unit polypeptides may be observed by SDS-PAGE (Redl *et al*, 1980).

It is important to demonstrate, when comparing the bond strength of Beriplast® to that of tTG, that the sealant is active and fibrin-monomer polymerisation is occurring.

Figure III-5 shows the fibrin sub-unit composition of Beriplast® fibrinogen solution with and without incubation with Beriplast® Thrombin/CaCl solution (at 37°C for 20 minutes) as analysed by SDS-PAGE.

Beriplast® fibrinogen solution, without incubation with Thrombin/CaCl solution, shows the α, β and γ sub-units of the fibrin monomers (Lane A Fig III-5, bands representing sub-units run α, β, γ in order of decreasing molecular weight).

Lane B contains Beriplast® fibrinogen solution after incubation with Beriplast® Thrombin/CaCl solution. Here the γ sub-unit band seen in Lane A is absent and an additional band, representing Factor XIIIa cross-linked γ-γ dimers, is present at a Mr of between 116 and 97.4 Kda.

These results show the sealant is active and also that a 20 minute incubation of the Beriplast® fibrinogen and thrombin solutions at 37 °C is sufficient time to allow fibrin sub-unit cross-linking (the formation of γ-γ dimers) and therefore provisional adhesion to occur.

Whilst a band indicative of α-α dimers is not visible in Lane B, this cross-linking reaction can take up to 24 hours for completion. Redl *et al* (1980) showed that even after α-chain cross-linking to a degree of 35% in fibrin sealant (after approximately a 10 min reaction time), 70% of maximum tensile strength is still achieved. So the lack of α-α dimers in the Beriplast® preparation following a 20-minute incubation time should not mean that Beriplast® bond strength is being compromised.

High Mr polymers of cross-linked fibrin are faintly visible at the top of Lane B, but their size makes entry into the resolving gel difficult.

SDS-PAGE analysis has thus shown that the sealant is active and that the incubation conditions used when analysing Beriplast® bond strength were sufficient for it to achieve a level of polymerisation that equated to it showing provisional adhesive strength.

III-3.3 Stress-strain plots for tTG and Beriplast® catalysed bonding of cartilage-cartilage interfaces

Figure III-6 shows a diagram of force vs. displacement plots obtained during the measurement of bond strength in response to tensile stress, for tTG and Beriplast®.

For both preparations, following an initial lag period, the plot shows an approximately linear relationship between force and displacement up to the yield point. For tTG-catalysed bonding, the force dropped rapidly once maximum load had been reached. After maximum loading of the Beriplast® treated sample, the decline in force was also steep although slightly more gradual than was found for the tTG treated sample.

Figure III-5 SDS-PAGE analysis of the fibrin monomer sub-unit composition of Beriplast® fibrinogen solution, with and without incubation with Beriplast® Thrombin/CaCl solution

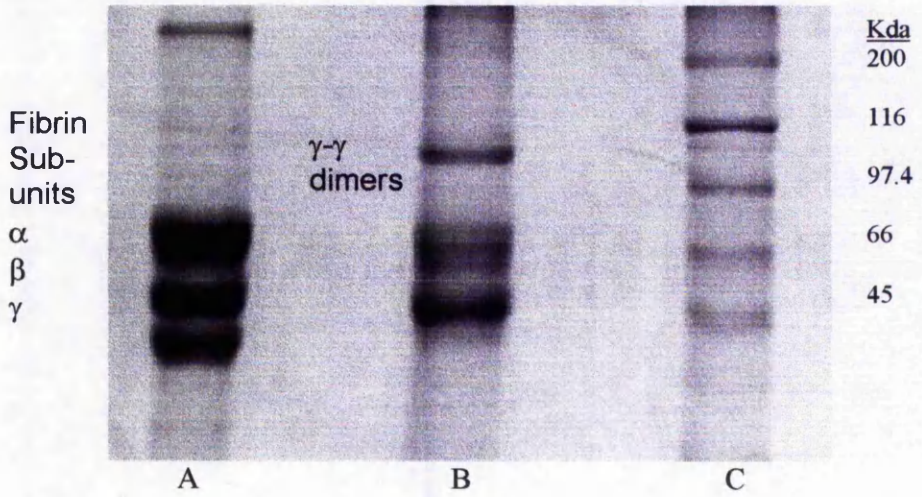


Figure III-5.

Beriplast® solutions analysed via SDS-PAGE on a 10% acrylamide content resolving gel. 5µl of sample or marker were loaded per lane (for method see III-2.2.2)

Lane A - Beriplast® fibrinogen solution without incubation with Beriplast® Thrombin/CaCl solution

Lane B - Beriplast® fibrinogen solution after a twenty-minute incubation with Beriplast® Thrombin/CaCl solution at 37°C

Lane C – Broad-range molecular weight markers (SIGMA)

Figure III-6. Diagrammatic representation of stress-strain curves (Force vs. displacement) obtained from measurement of tTG and Beriplast® bond strength at cartilage-cartilage interfaces in response to tensile stress

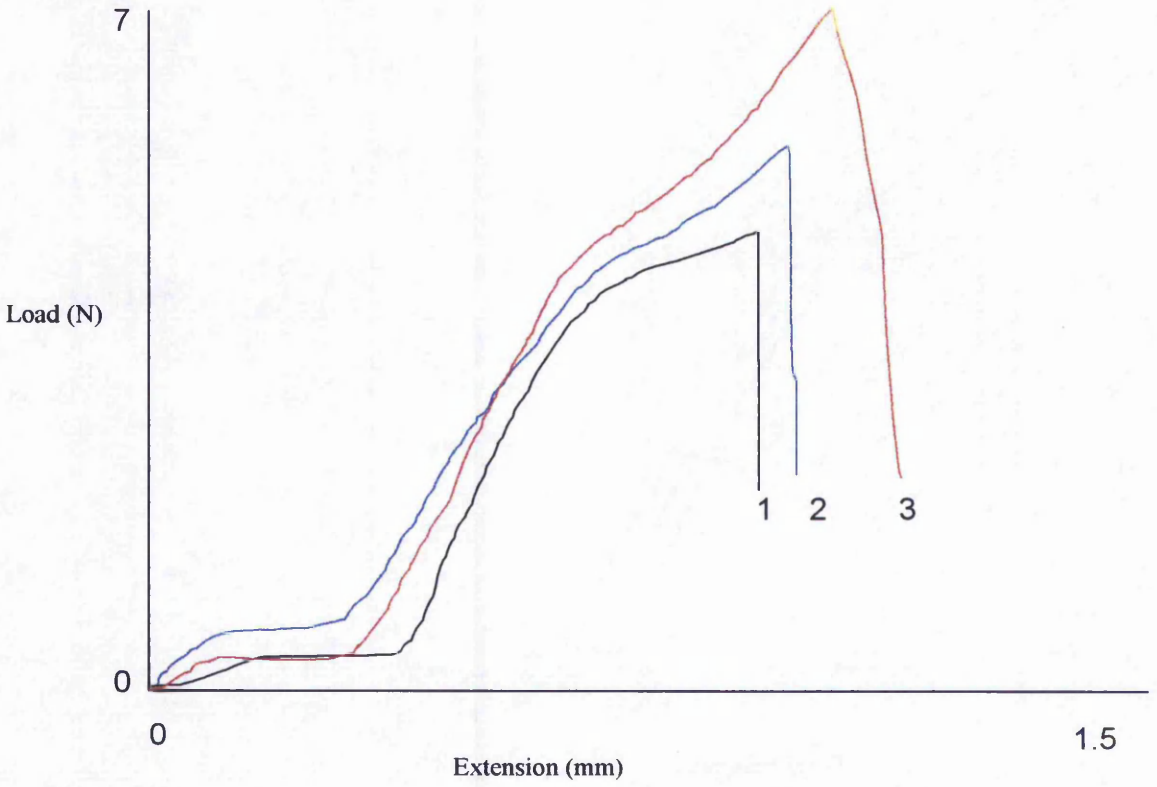


Figure III-6.

Measurement of bond strength in response to tensile stress of bonded cartilage-cartilage interfaces – Diagrammatic Load / Extension plots for interfaces bonded by:

1. tTG (8 mg/ml) preparation, maximum load 4.279 N
2. tTG (8 mg/ml) preparation, maximum load 5.383 N
3. Beriplast®, maximum load 6.809 N

All following incubation for 20 minutes at 37°C, 20% humidity (for methods see III-2.1.1 and III-2.2.1)

III-3.4 Bond strength of tTG at a cartilage-cartilage interface in the presence of reducing agent

Tissue transglutaminase is at its most active in its reduced form and there was concern that the tTG preparations being used may be becoming oxidised, losing activity and not achieving the maximum bond strength possible.

However, tTG bond strength at a cartilage-cartilage interface did not increase in the presence of DTT (a reducing agent), showing that the tTG preparations used thus far are not losing any bond strength due to tTG oxidation before application (Figure III-7). A bond strength of 0.047 ± 0.004 MPa was achieved with tTG (4mg/ml) alone, compared to 0.049 ± 0.002 MPa from an equivalent preparation in the presence of DTT (4mM, under standard incubation conditions).

III-3.5 Bond strength of tTG at pre-treated cartilage-cartilage interfaces

A feature of cartilage that defines its physiological role is its' anti-adhesive nature and this property becomes a hindrance when attempting to bond two cartilage surfaces together. Therefore pre-treatment of the tissue surfaces was investigated, with the intention of encouraging bonding at the interface by either reducing the anti-adhesive nature of the cartilage itself, or promoting increased tTG-catalysed cross-linking.

III-3.5.1 Effect of etching / scoring

Figure III-8 indicates that the bond strength of tTG was increased when the cartilage surfaces had been physically scored before application of enzyme solution, with incisions made in a grid-pattern 2 mm apart. A bond strength of 0.047 ± 0.004 MPa was recorded for tTG (4mg/ml) at an untreated interface, compared to 0.073 ± 0.005 MPa at a scored interface (a statistically significant, $p<0.05$, increase). The buffer control solution did not exhibit a similar increase in bond strength, suggesting that the increase found with use of tTG may have been due to the etching revealing more potential tTG substrate residues on the cartilage surface. Thus giving stronger adhesion by increasing the number of potential cross-links between the interface.

Figure III-7 Bond strength achieved at a cartilage-cartilage interface with use of tTG, with and without the presence of reducing agent, under standard incubation conditions

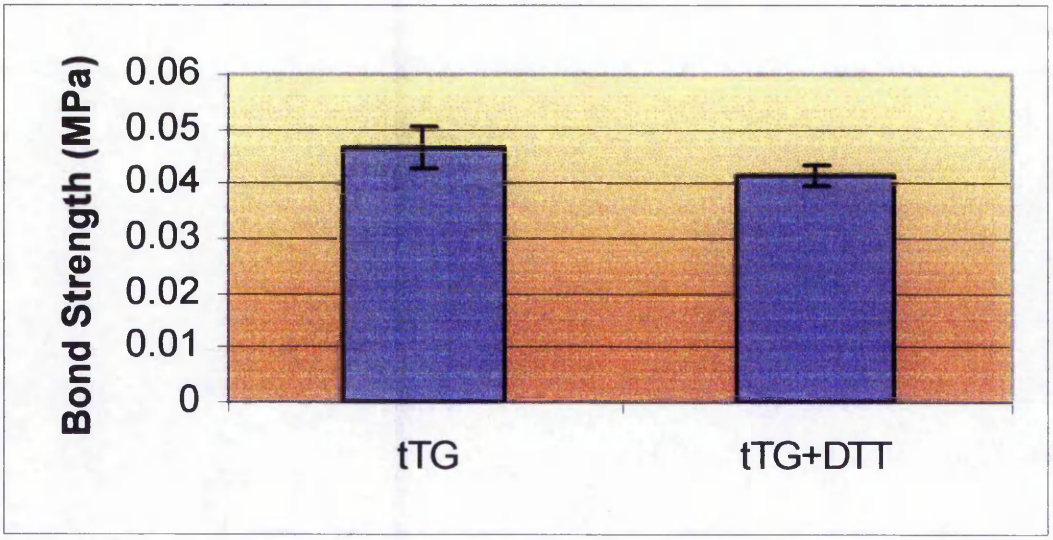


Figure III-7.

Bond strength of tTG (4mg/ml, 4172 nmol/hr/mg specific activity) with and without the presence of DTT (4mM) after incubation for 20min at 37°c, 20% humidity (for methods, see III-2.1.1). Figures show mean bond strength ± standard error (n=4).

Figure III-8 Influence of physically scoring the cartilage surfaces on the bond strength achieved at a cartilage-cartilage interface with use of tissue transglutaminase or buffer control, under standard incubation conditions

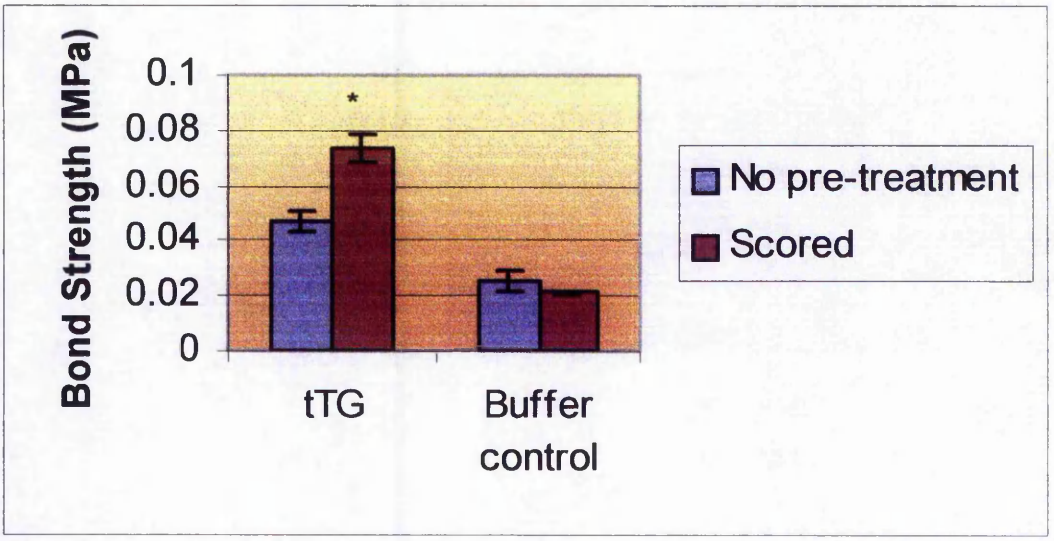


Figure III-8.

Bond strength of tTG (4mg/ml, 4172 nmol/hr/mg specific activity) and buffer control after incubation for 20min at 37°c, 20% humidity, at untreated cartilage-cartilage interfaces and interfaces pre-treated by being scored with a surgical scalpel prior to bonding humidity (for methods, see III-2.1.1 and III-2.3.1). Figures show mean bond strength ± standard error (n=4). Statistically significant data (scored compared to no pre-treatment within tTG treated samples) are marked as * ($p<0.05$).

III-3.5.2 Effect of drying

Tissue transglutaminase bond strength at an untreated tissue interface was compared to that shown by a similar enzyme preparation applied to a cartilage-cartilage interface whose surfaces had been heated for 5 minutes to remove any surface liquid. This drying of the cartilage surfaces led to a statistically significant ($p<0.05$) increase in the bond strength shown by both tTG and the buffer control at the tissue interface (Figure III-9).

For tTG (4mg/ml), bond strength increased from 0.054 ± 0.004 MPa at an untreated interface, to 0.085 ± 0.008 MPa after drying. The removal of surface water seems to be effective in promoting bonding between the cartilage surfaces, but how easy this drying would be to achieve in the water-saturated atmosphere of the body is open to question. Plus, it is likely that the removal of water from the tissue would have deleterious effects on the structure of the cartilage and the viability of its cells.

III-3.5.3 Effect of exposure to UVA irradiation

A further pre-treatment method investigated was exposure to UVA irradiation. UVA has been shown to increase the availability of tTG substrates within the extra-cellular matrix (Gross, 2000).

Exposure of interface surfaces to UVA irradiation for 20 minutes failed to lead to an increase in tTG bond strength (Figure III-10). A figure of 0.054 ± 0.04 MPa was achieved for tTG (4mg/ml) at an untreated interface (under standard incubation conditions) compared to 0.051 ± 0.002 MPa for tTG applied at an irradiated interface.

Figure III-9 Influence of drying the cartilage surfaces, on the bond strength achieved at a cartilage-cartilage interface with use of tTG or buffer control, under standard incubation conditions

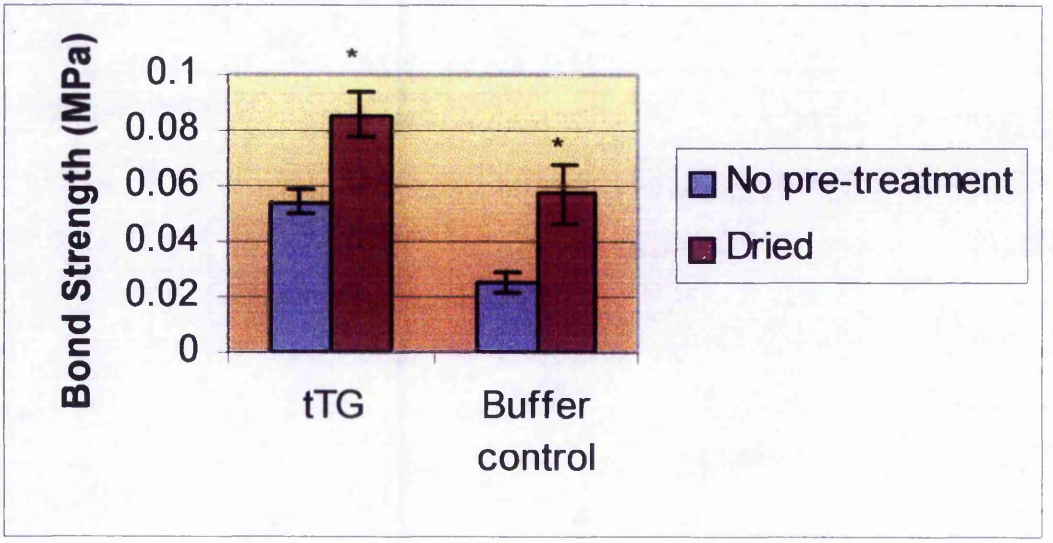


Figure III-9.

Bond strength of tTG (4mg/ml, 3522 nmol/hr/mg specific activity) and buffer control after incubation for 20min at 37°c, 20% humidity, at untreated cartilage-cartilage interfaces and interfaces pre-treated by being dried for 5 minutes prior to bonding humidity (for methods, see III-2.1.1 and III-2.3.2). Figures show mean bond strength ± standard error (n=4). Statistically significant data (dried compared to no pre-treatment within each group) are marked as * ($p<0.05$).

Figure III-10 Influence of exposure of cartilage surfaces to UVA irradiation, on the bond strength achieved at a cartilage-cartilage interface with use of tTG under standard incubation conditions

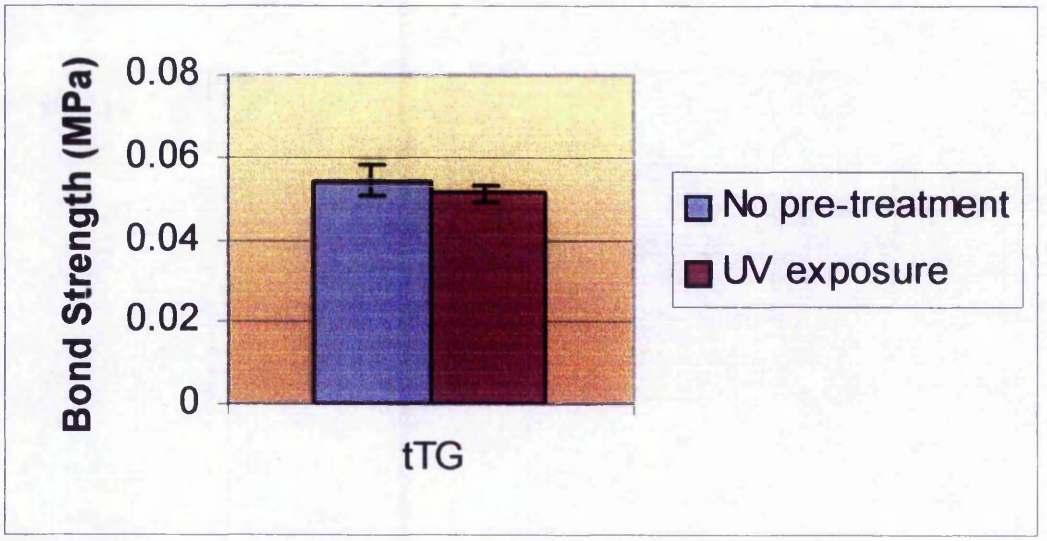


Figure III-10.

Bond strength of tTG (4mg/ml, 3522 nmol/hr/mg specific activity) after incubation for 20min at 37°c, 20% humidity, at untreated cartilage-cartilage interfaces and interfaces pre-treated by being exposed to UVA irradiation for 20 minutes prior to bonding humidity (for methods, see III-2.1.1 and 2.3.3). Figures show mean bond strength ± standard error (n=4).

III-3.5.4 Effect of the digestion of glycosaminoglycan (GAG) groups

Articular cartilage is rich in various types of proteoglycans (Morgelin *et al*, 1994) which give the tissue its unique gel-like properties and resistance to deformations (see I-2.1.3). The main structural component of the complex proteoglycans is hyaluronic acid, a negatively charged polysaccharide. Bound to it are core proteins and to these are bound glycosaminoglycans such as chondroitan sulphate, keratan sulphate and dermatan sulphate. These glycosaminoglycans are highly charged carbohydrate polymers that confer an anti-adhesive property to the cartilage surface. Removing proteoglycans from tissue surfaces is known to promote the attachment of repair materials (Hunziker & Rosenberg, 1996).

Chondroitinase AC (CAC) catalyses the depolymerization of chondroitan-4-sulphate and chondroitin-6-sulphate units within proteglycans and Jurgensen *et al* (1997) showed that controlled pre-treatment of cartilage surfaces with this enzyme increased the shear adhesive strength shown by tTG at a cartilage-cartilage interface. In this study, pre-treatments were performed to digest glycosaminoglycan groups from the surfaces of a cartilage-cartilage interface to reduce its anti-adhesive nature and promote tTG-catalysed bonding between the tissues.

Figure III-11 shows the effect on the bond strength of tTG at a cartilage-cartilage interface, after the tissue surfaces have been biochemically pre-treated with Chondroitinase AC (CAC). The bond strength of tTG (4mg/ml) at an untreated interface was 0.047 ± 0.004 MPa, which was significantly ($p<0.05$) increased upon when the cartilage surfaces were pre-treated for 5 minutes with CAC (1 U/ml), rising to 0.079 ± 0.010 MPa. A prolonged digestion (15 min) gave a further improvement in bond strength (0.099 ± 0.06 MPa, also a significant ($p<0.05$) increase compared to tTG at an untreated interface). The bond strength of tTG was not increased when the pre-incubation was performed using buffer as a control, which indicates that it was the action of CAC, not the environmental pre-incubation conditions, which gave rise to the increases described. The bond strength of the buffer control did rise slightly following the CAC pre-treatment, but not as much as for tTG. This seems to indicate that the enzyme is reducing the general anti-adhesive nature of the tissue surface,

probably by removing negative charge, and also having an additional effect that is causing the more dramatic increase in tTG bond strength.

Application of both CAC and tTG simultaneously does not give the increase in bond strength shown when the enzymes are used in sequence as previously described. When applied to an untreated interface, a solution containing tTG (4mg/ml) and CAC (1U/ml) produced a bond strength of 0.033 ± 0.007 MPa compared to 0.056 ± 0.008 MPa found for a solution of tTG (4mg/ml) alone (Figure III-12). It may be that the chondroitinase did not have time to digest the surface of the interface before tTG cross-linked it. Or the two enzymes may have inhibited one another's action.

Figure III-11 Bond strength of tTG and buffer control at cartilage-cartilage interfaces pre-treated to remove GAG-groups from the tissue surfaces, after incubation under standard conditions

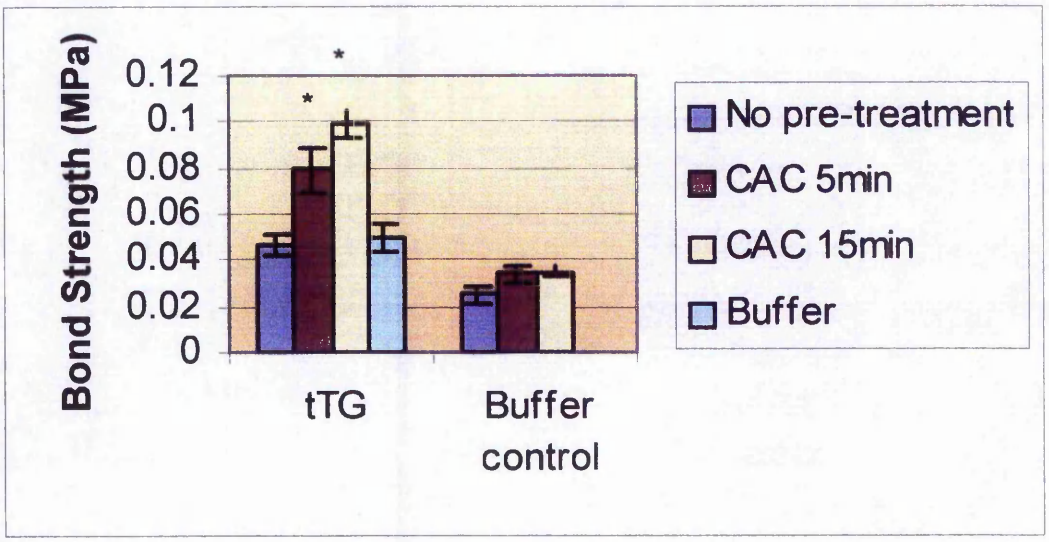


Figure III-11.

Bond strength of tTG (4mg/ml, 4172 nmol/hr/mg specific activity) and buffer control (after incubation for 20min at 37°C, 20% humidity) at untreated cartilage-cartilage interfaces, or interfaces digested with CAC (1U/ml) or buffer control for 5 or 15 min at 37°C prior to bonding humidity (for methods, see III-2.1.1 and III-2.3.4). Figures show mean bond strength ± standard error (n=4). Statistically significant data (compared to no pre-treatment within each group) are marked as * ($p<0.05$).

Figure III-12 Bond strength of tTG, with and without the presence of CAC, at cartilage-cartilage interfaces following incubation under standard conditions

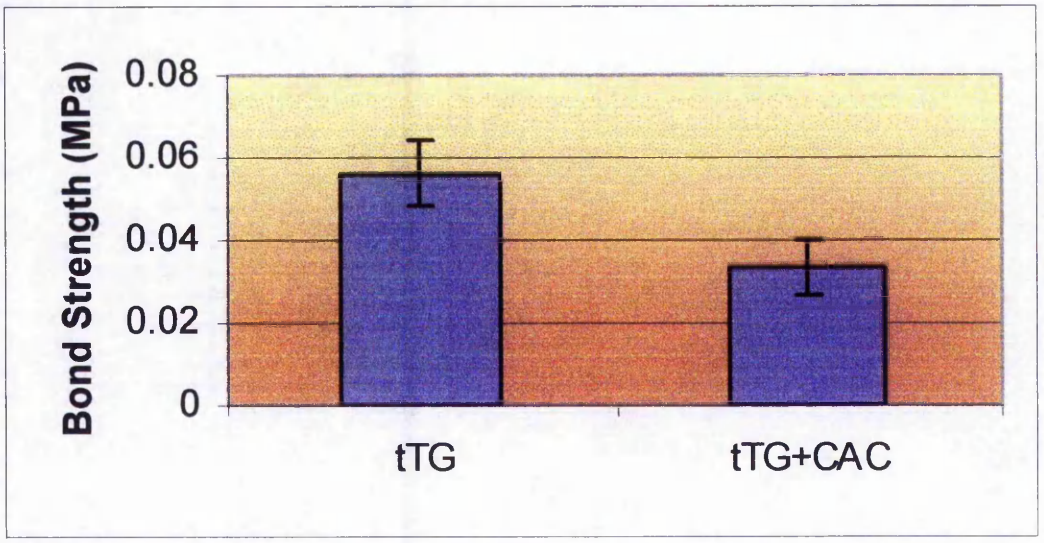


Figure III-12.

Bond strength of tTG (4mg/ml, 3084 nmol/hr/mg specific activity), with and without the presence of CAC (1U/ml), after incubation for 20min at 37°c, 20% humidity (for methods, see III-2.1.1 and III-2.3.4). Figures show mean bond strength ± standard error (n=4).

Further digestions were performed, using other enzymes known to depolymerise cartilage glycosaminoglycans, to see if these had the same effect on enhancing the bond strength of tTG as had the pre-treatment using chondroitnase AC.

Figure III-13 shows the influence on the bond strength of tTG (at a cartilage-cartilage interface) upon biochemically pre-treating the tissue surfaces with Hyaluronidase (Hya), Chondroitinase ABC (CABC) and Heparinase (Hep). All of which de-polymerise cartilage glycosaminoglycans.

When compared to application at an untreated interface, tTG bond strength (at 4mg/ml) increased from 0.029 ± 0.003 MPa to 0.056 ± 0.011, 0.059 ± 0.006 and 0.069 ± 0.004 MPa when the cartilage surfaces had been pre-treated for 15 minutes with Hyaluronidase, Chondroitinase ABC or Heparinase respectively (all at 1U/ml). These bond strengths were all statistically significant ($p<0.05$) increases compared to tTG at untreated interfaces.

Would a combined pre-treatment of several GAG-digesting enzymes at once have a cumulative effect on enhancing the bond strength of tTG? .

The bond strength of tTG at a cartilage-cartilage interface was determined after the tissue surfaces had been bio-chemically pre-treated with a solution of Hyaluronidase / Chondroitinase ABC / Heparinase and Chondroitinase AC (1 U/ml solution with respect to each enzyme) for 15min at 37°C and 20% humidity.

Bond strength increased from 0.043 ± 0.003 MPa for tTG at an untreated cartilage-cartilage interface, to 0.101 ± 0.009 MPa at an interface subjected to the combined GAG-digestion (Figure III-14).

Whilst this multiple digestion led to a significant ($p<0.05$) increase in tTG bond strength, the size of the increase (from that found at an untreated interface to that measured at a digested interface) was the same for the combined digestion as it was for the single Heparinase digestion previous. It seems therefore that the combined digestion is no more beneficial to the bond strength of tTG than a pre-treatment using one GAG-digesting enzyme alone.

Figure III-13 Bond strength of tTG at cartilage-cartilage interfaces pre-treated to remove GAG-groups from the tissue surfaces, after incubation under standard conditions

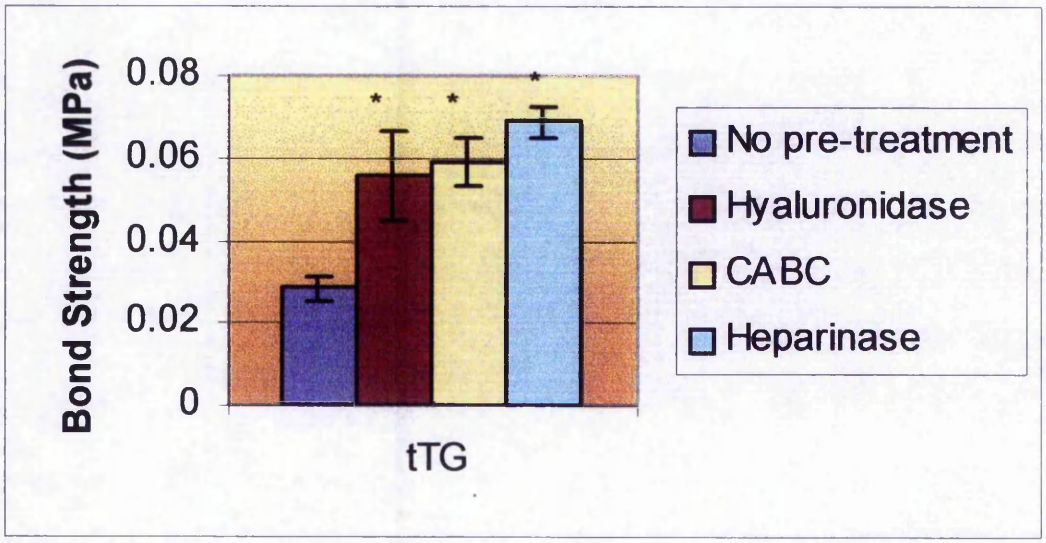


Figure III-13.

Bond strength of tTG (4mg/ml, 3825 nmol/hr/mg specific activity, after incubation for 20min at 37°C, 20% humidity) at untreated cartilage-cartilage interfaces and interfaces pre-treated with Hyaluronidase, Chondroitinase ABC or Heparinase prior to bonding (all 1U/ml for 15 minutes at 37°C) (for methods, see III-2.1.1 and III-2.3.4). Figures show mean bond strength ± standard error (n=4). Statistically significant data (compared to no-pre-treatment) are marked as * ($p<0.05$).

Figure III-14 Bond strength of tTG at cartilage-cartilage interfaces pre-treated to remove GAG-groups from the tissue surfaces, after incubation under standard conditions

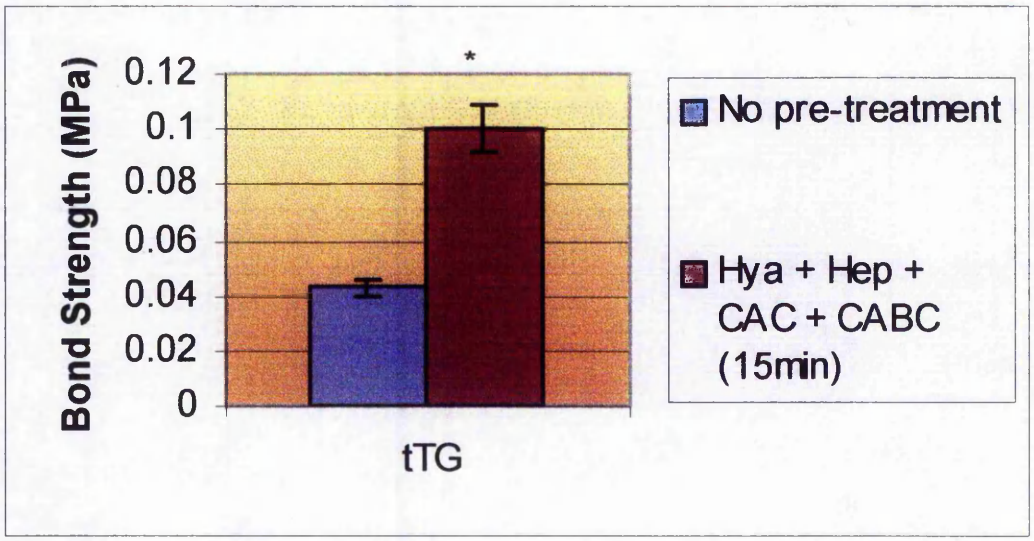


Figure III-14.

Bond strength of tTG (4mg/ml, 3140 nmol/hr/mg specific activity, after incubation for 20min at 37°C, 20% humidity) at untreated cartilage-cartilage interfaces and interfaces pre-treated with a combined solution of Hyaluronidase, Heparinase, Chondroitinase AC and Chondroitinase ABC prior to bonding (all 1U/ml for 15 minutes at 37°C) (for methods, see III-2.1.1 and III-2.3.4). Figures show mean bond strength ± standard error (n=4). Statistically significant data (compared to no pre-treatment) are marked as * ($p<0.05$).

III-3.5.5 Effect of the digestion of collagen

The main structural components of cartilage are collagen fibres, termed 'Type II' in collagen nomenclature.

The controlled pre-treatment of tissue surfaces with a crude-collagenase (EC 3.4.24.3) was also performed, in order to see if this would increase the bond strength shown by tTG at a cartilage-cartilage interface (as occurred after GAG digestion).

However, collagenase pre-treatment actually led to a significant ($p<0.05$) reduction in the bond strength shown by tTG at the tissue interface (Figure III-15).

Bond strength fell from 0.050 ± 0.005 MPa, for tTG (4mg/ml) at an untreated interface, to 0.023 ± 0.001 MPa for an equivalent preparation at an interface that had been digested for 20min with a collagenase solution (20U/ml).

Digestion of collagen may have had an adverse affect on the bond strength of tTG by removing potential tTG cross-linking sites from the tissue surfaces. Collagenase digestion of the structural collagen fibres could have led to less TG-substrates being available at the tissue interface.

III-3.5.6 Effect of the digestion of GAG's and collagen

When collagenase digestion is followed by GAG-digestion, then the bond strength of tTG at such an interface is equivalent to that which it shows at a GAG-digested interface alone. There is no reduction in bond strength, as seen following the collagenase digestion.

Figure III-16 shows that tTG (4mg/ml) bond strength rose from 0.043 ± 0.008 MPa at an untreated interface, to 0.104 ± 0.014 MPa at an interface digested for 15min using CAC (1U/ml). When the CAC digestion was proceeded by a collagenase digestion, tTG showed a bond strength of 0.110 ± 0.009 MPa (5min collagense digestion) and 0.100 ± 0.015 MPa (15min collagenase digestion). All of the bond strengths found at treated interfaces were statistically significant ($p<0.05$) increases compared to the bond strength found for tTG at untreated interfaces.

This suggests that the subsequent digestion of GAG's compensates for the adverse effects of the collagenase digestion.

Figure III-15 Bond strength of tTG at cartilage-cartilage interfaces pre-treated to digest collagen from the tissue surfaces, after incubation under standard conditions

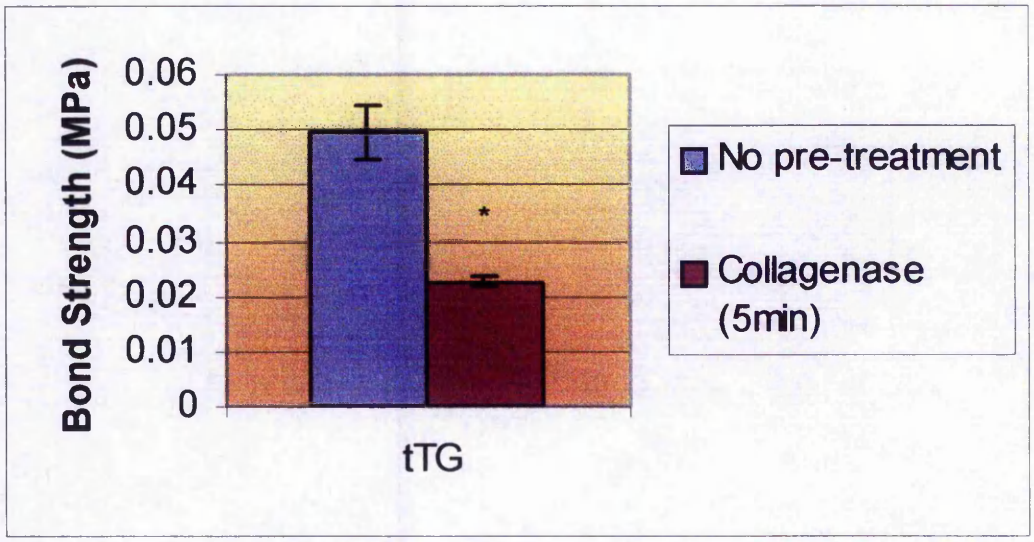


Figure III-15.

Bond strength of tTG (4mg/ml, 3544 nmol/hr/mg specific activity, after incubation for 20min at 37°C, 20% humidity) at untreated cartilage-cartilage interfaces and interfaces pre-treated with collagenase (from *Clostridium histolyticum*) prior to bonding (20U/ml for 20 minutes at 37°C) (for methods, see III-2.1.1 and III-2.3.4). Figures show mean bond strength ± standard error (n=4). Statistically significant data (compared to no pre-treatment) are marked as * ($p<0.05$).

Figure III-16 Bond strength of tTG at cartilage-cartilage interfaces pre-treated to remove GAG-groups and digest collagen from the tissue surfaces, after incubation under standard conditions

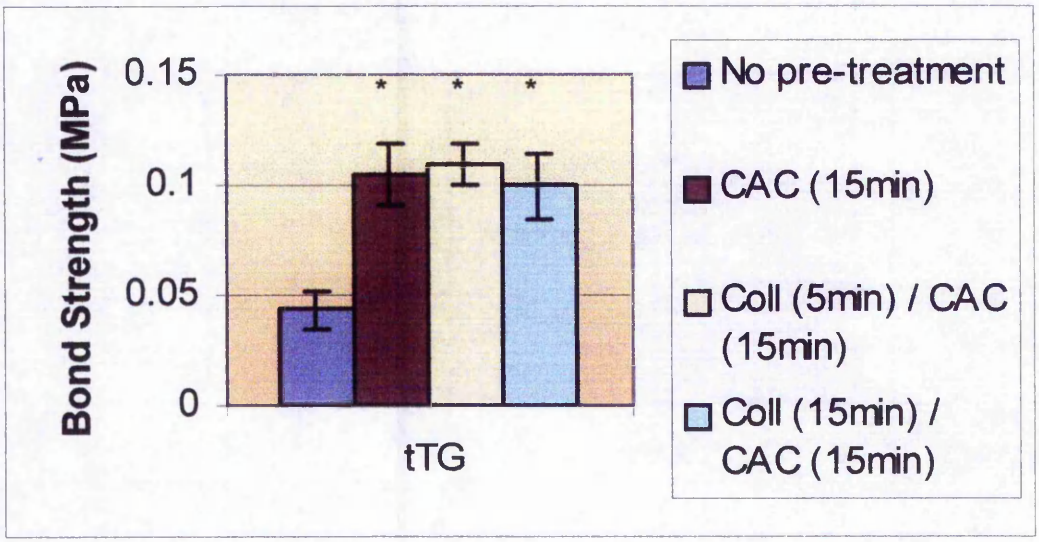


Figure III-16.

Bond strength of tTG (4mg/ml, 3185 nmol/hr/mg specific activity, after incubation for 20min at 37°C, 20% humidity) at untreated cartilage-cartilage interfaces and interfaces pre-treated with Chondroitinase AC (1U/ml, 15 minutes at 37°C) or Collagenase (from *Clostridium histolyticum* , 20U/ml, 5 or 15 min at 37°C) followed by Chondroitnase AC (1U/ml for 15 min at 37°C) prior to bonding (for methods, see III-2.1.1 and III-2.3.4). Figures show mean bond strength ± standard error (n=4). Statistically significant data (compared to no pre-treatment) are marked as * ($p<0.05$).

III-3.5.7 Bond strength of tTG at physically and biochemically pre-treated cartilage-cartilage interfaces – Effect of scoring and GAG-digestion combined

Two of the surface pre-treatments found to raise tTG bond strength at the cartilage-cartilage interfaces were the physical scoring of the tissue and the digestion of GAG groups. These were combined to see if the two treatments could have a combined effect in raising tTG bond strength.

Cartilage surfaces were either left untreated, scored (as described previous), digested for 15min at 37°C with CAC (1U/ml), or both scored and digested. Figure III-17 shows the bond strength of tTG (4mg/ml) at each interface type. When applied to the untreated interface, tTG gave a bond strength of 0.056 ± 0.011 MPa. This rose to 0.084 ± 0.011 MPa at a scored interface, 0.089 ± 0.014 MPa at a digested interface and 0.095 ± 0.004 MPa at an interface receiving both treatments.

The bond strength of tTG was no greater at an interface that was both etched and CAC-digested, as it was at interfaces that received one treatment alone. But both treatments raised the bond strength of tTG compared to that of the control interface, as found previously (Fig III-8, Fig III-11).

Whilst the only statistically significant ($p<0.05$) increase in bond strength was found following the combined pre-treatment (compared to tTG bond strength at untreated interfaces), the bond strength found for this particular batch of tTG at untreated interfaces was unusually high.

Figure III-17 Bond strength of tTG at cartilage-cartilage interfaces pre-treated via scoring, digestion of GAG groups or both, under standard conditions

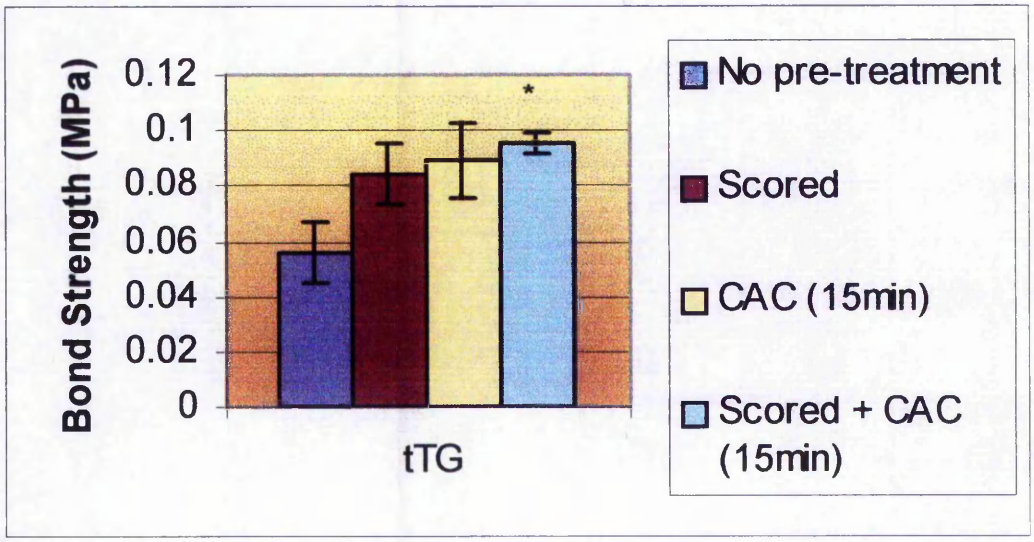


Figure III-17.

Bond strength of tTG (4mg/ml, 2033 nmol/hr/mg specific activity, after incubation for 20min at 37°C, 20% humidity) at untreated cartilage-cartilage interfaces and interfaces which had been either scored with a surgical scalpel, digested with Chondroitinase AC (1U/ml for 15min at 37°C) or both scored and digested, prior to bonding (for methods, see III-2.1.1 and III-2.3.4). Figures show mean bond strength ± standard error (n=4). Statistically significant data (compared to no pre-treatment) are marked as * ($p<0.05$).

III-4 Conclusions

The tensile bond strength of tTG at a cartilage-cartilage interface, as determined using the novel *in vitro* test method, exhibited an approximately linear dependence on enzyme concentration. When applied in the presence of EDTA (tTG inhibitor), tTG bond strength reduced to a level similar to that of the control solution, indicating that the enzyme is playing an active role in the adhesion process.

An 8mg/ml tTG preparation showed comparable bond strength to that of a commercially available fibrin sealant (Beriplast®) under equivalent incubation conditions and the bond strength of both glues could be enhanced by increasing the duration of incubation from 10 to 20 minutes and again from 20 to 40 minutes. The control solution did not show a similar time-dependence, which suggests that the trend of increasing bond strength of tTG solutions is not merely due to samples drying out along the cartilage-cartilage interface.

Tissue transglutaminase showed consistent bond strength over an incubation humidity range of 20-100% where as Beriplast® showed a marked reduction in adhesive capabilities when incubation conditions exceeded 90% humidity. This indicates that tTG should maintain its gluing capacity if applied in the fluid-filled atmosphere of the body.

Force vs. displacement plots for tTG and Beriplast® bonding of cartilage-cartilage interfaces showed an approximately linear increase in force to the point of failure. For both preparations this followed an initial lag period, which may be due to a property of the test system (e.g. samples slipping slightly within the clamping devices) or of the tissue. Testing of the tensile strength of certain materials also produces a period where strain lags behind the stress, termed anelastic deformation. Which is the result of time dependent processes such as the straightening out of coiled molecular chains within the material, or the capacity of the material to damp out vibrations (Anderson *et al* 1990). The linear nature of the stress-strain curve up to the point of failure was also seen for tTG and fibrin sealant catalysed bonding of cartilage-cartilage interfaces in response to shear stress (Jurgensen *et al*, 1997). As well as fibrin sealant bonding of cartilage-cartilage and cartilage-periosteum interfaces in response to shear stress (Orr *et al*, 1999). After maximum load had been reached, the decline in the stress-strain

curve for Beriplast® bonding was slightly less steep than found for tTG. But not as gradual as was seen in the other studies (Jurgensen *et al* 1997; Orr *et al*, 1999). This multiphase decline (compared to the sharp decline seen in tTG plots) has been attributed to the more complex patterns of interaction that occur during fibrin sealant bonding (non-covalent fibrin polymerisation and covalent FXIIIa-catalysed cross-linking) compared to tTG-catalysed bonding (Jurgensen *et al*, 1997).

Fibrin adhesives have been evaluated under many different test conditions, often using formulations individual to the authors, which makes comparisons of the bond strengths shown difficult. Sealant composition and full details of test methods are rarely reported in the literature. Those preparations with higher fibrin concentrations generally give higher adhesive strengths (Redl *et al*, 1980), so variations in the adhesive strengths given in the literature are most likely due to varying fibrin concentrations.

Claes et al (1980) reported tensile testing of a commercial fibrin adhesive at a cartilage-cartilage interface (the method most relevant to that reported here) and found an adhesive strength of 7.1 N/cm^2 for a commercial fibrin adhesive. This is equivalent to ~ 0.071 MPa and correlates well with the adhesive strength found for Beriplast® using our novel method.

The trends in the tensile bond strength of tTG, mirror the shear adhesive strength findings of Jurgenson et al (1997), and indicate that tTG has the potential to bond a cartilage-cartilage interface in response to a variety of stresses and strains. Also, the bond strength found here for Beriplast® falls within the range of those reported for fibrin sealant, which seems to validate the novel test method as one which produces results that are consistent with the findings of others.

Pre-treatment of cartilage surfaces prior to bonding, via digestion of glycosaminoglycans, enhanced the tensile bond strength of tTG at the tissue interface. This increase was time-dependent with respect to the duration of digestion. Similar pre-treatment with collagenase led to a reduction in tTG bonding, possibly due to it removing tTG substrates from the bonding surfaces. The digestion of collagen and GAG's in succession did not lead to any improvement in bond strength compared to GAG digestion alone. Digestion of GAG's has been shown to promote the attachment of repair materials to cartilage (Hunziker & Rosenberg, 1996), possibly by removing

negative charge from the tissue surface. Investigation into why GAG-digestion promotes tTG-catalysed bonding of cartilage surfaces (also reported by Jurgensen et al (1997)) is discussed in Chapter IV.

Drying of the cartilage surfaces, for 5 minutes prior to bonding, raised the bond strength shown by tTG and the buffer control solution at the treated interface. Any greater length exposure of the tissue surfaces to the drying lamp led to gross morphology changes and 'melting' of the cartilage. How practical even five minutes drying of the tissue (pre-tTG application) would be to the *in vivo* adhesive-user remains to be seen. Nor any possible adverse affects on chondrocyte viability and cartilage structure due to the removal of water from the tissue.

Exposure of tissue surfaces to UVA radiation as a pre-treatment method was not successful in raising the bond strength shown by tTG.

Physical scoring of the tissue surfaces helped increase tTG bond strength but not that of the control solution. This suggests that the scoring may have been revealing increased TG substrate sites on the cartilage surface for tTG to cross-link. But, as with the drying pre-treatment, the effects of physically cutting the cartilage would be likely to have an adverse effect on chondrocyte viability and cartilage structural integrity. A combination treatment of GAG digestion and scoring appeared no more beneficial to tTG bond strength than one pre-treatment alone.

Chapter IV : Analysis of cartilage surfaces

Chapter IV : Analysis of cartilage surfaces

IV-1 Introduction

Previous studies have shown that the enzymatic digestion of glycosaminoglycans (GAG's) from cartilage surfaces promotes the attachment of repair materials and aids tTG-catalysed bonding at a cartilage-cartilage interface (Hunziker & Rosenberg, 1996; Jurgensen *et al*, 1997). Work presented in this thesis has also demonstrated that GAG-digestion aids bonding of a cartilage-cartilage interface by tTG (see Chapter III).

GAG-digestion removes negative charge from the cartilage surface, lowering its anti-adhesive properties, and it has also been theorised that the digestion exposes more tTG-substrate residues on the tissue surface (Jurgensen *et al*, 1997).

In the present study, the surfaces of cartilage samples were analysed to determine what changes (topographical or biochemical) occurred following GAG-digestion, that might aid tTG-catalysed bonding.

A fundamental problem in analysing cartilage is examining the tissue in its native state, as cartilage is sensitive to many tissue preparation techniques because of its high water content, the removal of which can fundamentally alter the structure of the tissue.

Environmental Scanning Electron Microscopy (ESEM) removes the high vacuum constraint on the sample environment that is present in conventional scanning electron microscopy (SEM), allowing wet, oily, dirty, non-conductive samples to be examined in their natural state without modification or preparation. Cartilage, a specimen that must remain hydrated in order to retain its structure, therefore need not be dried before viewing via ESEM. Both environmental and 'cryostat' scanning electron microscopy were used here for cartilage surface analysis. 'Cryostat' SEM refers to a technique in which the tissue samples are flash-frozen in liquid nitrogen and viewed using a cryo-stage attachment, thus also intending to rapidly preserve the tissue in its natural, hydrated state.

A further technique used for surface structure analysis was Atomic Force Microscopy (AFM). This technique can image the 3-D surface structure of biological specimens in a fluid environment, without the need for destructive tissue preparation techniques (for review see Lal & John, 1994). Thus providing the means to observe biochemical and

physiological processes in real time, in an environment that maintains the specimen in as near as life-like condition as possible.

In addition to studying the surface topography of cartilage samples, the availability of tTG-substrate residues on GAG-digested and undigested cartilage surfaces was estimated by looking at the degree of tTG-catalysed incorporation of fluorescent TG-substrate molecules into the tissue (using Confocal Laser Scanning Microscopy).

An increased incorporation of fluorescein cadaverine (fluorescent TG substrate primary amine) into a tissue surface will indicate a greater number of potential TG-substrate glutamine residues being available thereupon (since cadaverine is a competitive primary amine substrate of TG and can be incorporated into peptide-bound γ-glutamine residues instead of peptide-bound lysine). The technique has previously been used to detect amine acceptor protein substrates accessible to transglutaminase expressed by rabbit articular chondrocytes (Lajemi *et al*, 1998) and fibroblasts (Verderio *et al*, 1998).

GAG-digestion at the cartilage-cartilage interface may be revealing increased numbers of tTG-substrate residues that, in turn, allow for the increased bond strength shown by tTG at digested interfaces by permitting increased cross-linking between the tissues.

Confocal laser scanning microscopy was also used for studying the topography of cut cartilage surfaces, by recording the variation in reflection of a laser beam upon the tissue surface and using this as a measure of its surface features.

IV-2 Materials and methods

IV-2.1 Scanning Electron Microscopy (SEM)

Cartilage samples (taken from porcine rib see II-2.1) were thawed at room temperature and cut into sections using a surgical scalpel. These sections took the form of discs of tissue, approximately 2-3mm thick. The cut surface of these cartilage samples were analysed on a Hitachi S-2500 Scanning Electron Microscope, fitted with a cryo-stage attachment (Oxford Instruments, Oxford, UK) at the British Leather Research Centre (Northampton, UK) with the assistance of Amanda Bugby. The samples were flash-frozen in liquid nitrogen and the cryo-stage attachment of the SEM ensured that they remained frozen during viewing.

Samples viewed were:

1. An untreated cartilage surface
2. A surface subjected to GAG-digestion.
   20µl of Chondroitinase AC (1U/ml in 50mM Tris.Cl (pH 7.3)) was applied to the surface of a cartilage sample, which was then incubated at 37°C for 5 minutes before being rinsed in PBS, flash-frozen and imaged as stated.

IV-2.2 Environmental scanning electron microscopy (ESEM)

Cartilage samples (taken from porcine rib see II-2.1) were thawed at room temperature and cut into sections using a surgical scalpel. These sections took the form of discs of tissue, approximately 2-3mm thick. The cut surface of these cartilage samples were analysed on a Phillips XL30 Environmental Scanning Electron Microscope-FEG at the University of Nottingham Material Engineering and Design Department, with the assistance of Nicola Bock.

Unlike SEM, samples need not be dried before being viewed using ESEM, as it provides a saturated water vapour environment that keeps samples fully hydrated. It also eliminates the need for conductive coatings and most other sample preparation techniques, which delicate structures often do not survive, and the need for the sample to be vacuum tolerant. In short, ESEM allows samples to be viewed in their natural state.

Samples viewed were:

1. An untreated cartilage surface
2. A 'Tested sample'.

   A cartilage sample prepared for adhesive strength testing (see II-2.2), separated manually and the interface surfaces viewed as stated.
3. A surface subjected to GAG-digestion.

   20 µl of Chondroitinase ABC (1 U/ml) was applied to the surface of a cartilage sample, which was then incubated at 37°C for 15min before being rinsed in PBS and imaged as stated.

IV-2.3 Confocal Laser Scanning Microscopy using laser reflection

Cartilage samples (taken from porcine rib see II-2.1) were thawed at room temperature and cut into sections using a surgical scalpel. These sections took the form of discs of tissue, approximately 2-3mm thick.

Samples viewed were:

1. A sample subjected to GAG-digestion

   20µl of Chondroitinase AC (1U/ml) was applied to the surface of the disks, which were then incubated in a humidity chamber at 37°C for 15 or 60 minutes. The surfaces were rinsed in PBS and blotted with filter paper before viewing.
2. An undigested sample which received 50mM Tris.Cl buffer (pH 7.3) in place of Chondroitinase AC solution.

Samples were viewed using a LEICA TCS-NT Confocal microscope (LEICA, Milton Keynes, UK) in reflection mode, with a x10 objective.

IV-2.4 Atomic Force microscopy (AFM)

AFM was performed using a Topometrix Explorer AFM with the assistance of Alan Brain at the Smith & Nephew Group Research Centre, York.

Cartilage samples (taken from porcine rib see II-2.1) were thawed at room temperature and cut into sections using a surgical scalpel. These sections took the form of discs of tissue, approximately 2-3mm thick, which were placed onto the AFM stage.

The tissue surface was kept moist throughout under 50mM Tris.Cl buffer (pH 7.3), which it retained on its surface. The tissue surface was analysed with the microscope in non-contact mode and the AFM stylus within the buffer, keeping the tissue surface permanently hydrated.

The cartilage surface was imaged under buffer using various scan sizes, giving an analysis of an untreated surface before GAG-digestion. The buffer was then removed and replaced with Chondroitinase AC solution (1U/ml) for 1 minute at room temperature. After removing the enzyme solution, the cartilage was rinsed 5x in buffer and re-imaged. This gave an image of the same region of surface as previous, but following a one-minute GAG-digestion. The process was repeated to give a further image of the area after a total of 2 minutes GAG-digestion.

IV-2.5 *In situ* incorporation of fluorescent TG substrates into cartilage surfaces - a measure of available peptide-bound γ-glutamyl groups for tTG cross-linking

Cartilage tissue samples were cut into small, rectangular block sections using a surgical scalpel, embedded in OCT tissue embedding medium (BDH Lab supplies, Poole, UK) on a steel cryostat chuck and snap frozen in liquid nitrogen. Sections (10μm thick) were then cut using a cryotome at -18°C. Sections were adhered to Superfrost Plus Gold slides (BDH Ltd, Poole, UK) and stored at -70°C until use.

Sample slides were thawed at room temperature and each tissue section circled using a hydrophobic pen (Vector Laboratories Inc, USA), before being briefly washed three times in 50mM Tris.Cl buffer (pH 7.3).

Each section was then treated with 40μl of CABC solution (1U/ml, buffer for controls) for 5 or 15 minutes at 37°C (in a slide humidity chamber). Sections were then washed in buffer before the addition of:

5μl 50mM Tris.Cl buffer (pH 7.3)

5μl 50mM CaCl solution (or 500mM EDTA solution or 200μm cystamine solution)

1.3μl of Fluorescein cadaverine (0.5mM in 10mM Tris.Cl pH 7.3) (Molecular Probes, Oregon, USA)

5μl of tTG (2mg/ml) or 5μl 50mM Tris.Cl buffer (pH 7.3) (control)

All sections were then incubated in a humidity chamber at 37°C for 60min. Followed by three washes in buffer, and two in ice-cold methanol. Sections were then mounted

in fluorescent mounting medium (Vectashield, Vector Laboratories Inc, USA) and stored at –20°C until viewing. Samples were viewed using a LEICA TCS-NT Confocal microscope with the argon krypton laser adjusted for fluorescein excitation, using a x40 objective

IV-3 Results

IV-3.1 Analysis of cartilage surfaces by Scanning Electron Microscopy (SEM)

Cartilage surface topography was examined using frozen samples viewed via a Scanning Electron Microscope fitted with cryo-stage attachment. Use of this method meant that the samples need not be dried, as in conventional SEM analysis, which may alter surface structures.

Figure IV-1 shows SEM images of cut cartilage surfaces at varying magnifications. The surfaces of both digested and undigested samples show multiple circular features and an undulating topography at lower magnification (x50, Figure IV-1 A and B).

At higher magnifications (x1000), multiple chain-like structures become apparent on both surfaces (Figure IV-1 C and D). These chains appear to be randomly orientated on the tissue surface and may be attributable to long-chain proteoglycan molecules.

The 'treated' surface was digested with Chondroitinase AC (1 U/ml, 15min) prior to viewing. This digestion of the cartilage surface, to remove GAG groups, appears to lead to no changes in the surface topography of the tissue as analysed by SEM. With both the treated and untreated surfaces having similar surface features. If the chain-like structures are indeed proteoglycans, then the digestion of their glycosaminoglycan constituent does not seem to produce any changes in their structure which are apparent when viewed using SEM, even at highest magnification (x5000, Figure IV-1 E and F).

Studies of cartilage surfaces have tended to concentrate on articular cartilage and the surface that is presented at articular joints. The surface of adult articular cartilage *en face* has shown the presence of multiple bowl-shaped surface depressions of microscopic size, similar to those seen in Figure IV-1 A & B. Mound-like elevations have also been reported in other studies of normal cartilage (Freeman, 1979).

Clarke and co-workers (1971) noted that these bowl-shaped depressions (shown by SEM of the articular surface of adult human cartilage) were similar in size and pattern to cell lacunae as shown in specimens from which a thin strip of the cartilage surface had been removed. He concluded that these depressions were due to the presence of underlying lacunae. Results suggested the cartilage surface to be undulating and contain, 'tertiary irregularities', due to the presence of the multiple hollows/prominences of 30-45μm in diameter.

Figure IV-1 Analysis of cut, hyaline, costal cartilage surfaces using Scanning Electron Microscopy, with and without prior digestion of glycosaminoglycan groups from the tissue surface

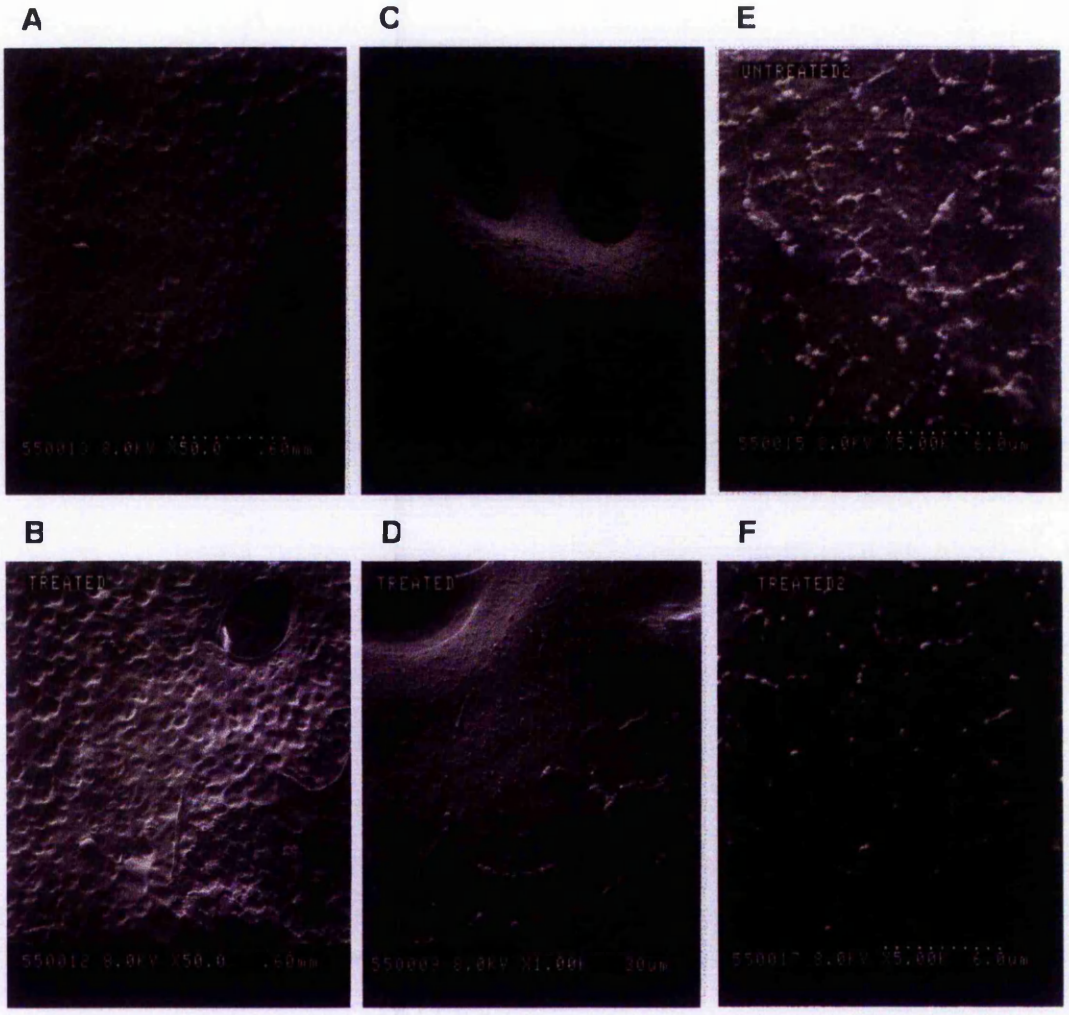


Figure IV-1.

Fig IV-1 A, C, E – Untreated cut cartilage surfaces viewed using frozen samples analysed by SEM, magnification x50, x1000, x5000 respectively

Fig IV-1 B, D, F – GAG-digested cut cartilage surfaces viewed using frozen samples analysed by SEM, magnification x50, x1000, x5000 respectively. GAG-digestion occurred using CAC (1U/ml) for 15min at 37°C prior to viewing (see IV-2.1)

IV-3.2 Analysis of cartilage surfaces by Environmental Scanning Electron Microscopy (ESEM)

The SEM analysis of cut cartilage surfaces used previously (IV-3.1) endeavoured to image the samples without the risk of possible alteration to their surface structures through drying or pre-coating. Viewing flash-frozen samples on a cryo-stage attachment did this. However, the freezing process meant that the tissue was still in some way altered before viewing and was not imaged in its natural state. To this end ESEM was used to study cut cartilage surfaces, which allowed them to be viewed hydrated and without preparation.

Figure IV-2 shows ESEM images of cut cartilage surfaces at varying magnifications. The surface of the cartilage again appears to contain multiple, circular features (between 20-40 μm in diameter), many of which are of a 'figure of eight' shape (Figure IV-2 A, B, C, D, E). This is consistent with the findings of SEM analysis and these features have previously been attributed to chondrocyte lacunae (see IV-3.1).

Hyaline cartilage cells vary in shape and are often found in-groups of two or more, which are the offspring of a common parent chondroblast. Such cells have a straight outline where opposed to each other, but a rounded contour in general (as seen in Fig IV-2) (Williams & Warwick, 1980).

A cartilage-cartilage interface that has been bonded using tTG (4 mg/ml) and then physically separated shows no change in surface topography due to tTG action (Figure IV-2 C & D compared to untreated surfaces, Fig IV-2 A, B). The circular features apparent in the untreated samples are still visible and the clusters of geometric shapes shown are probably attributable to salt crystals remaining from the applied activating buffer.

Digestion of a cartilage surface, to remove GAG groups, appears to lead to no changes in the surface topography of the tissue as analysed by ESEM.

Figure IV-2 E & F show that the surface of a cartilage sample, pre-treated with Chondroitinase ABC (1U/ml) for 15 minutes prior to viewing, shows no topological differences to the untreated samples shown in Figure IV-2 A, B.

Figure IV-2 Analysis of cut, hyaline, costal cartilage surfaces using Environmental Scanning Electron Microscopy, with and without prior digestion of glycosaminoglycan groups from the tissue surface

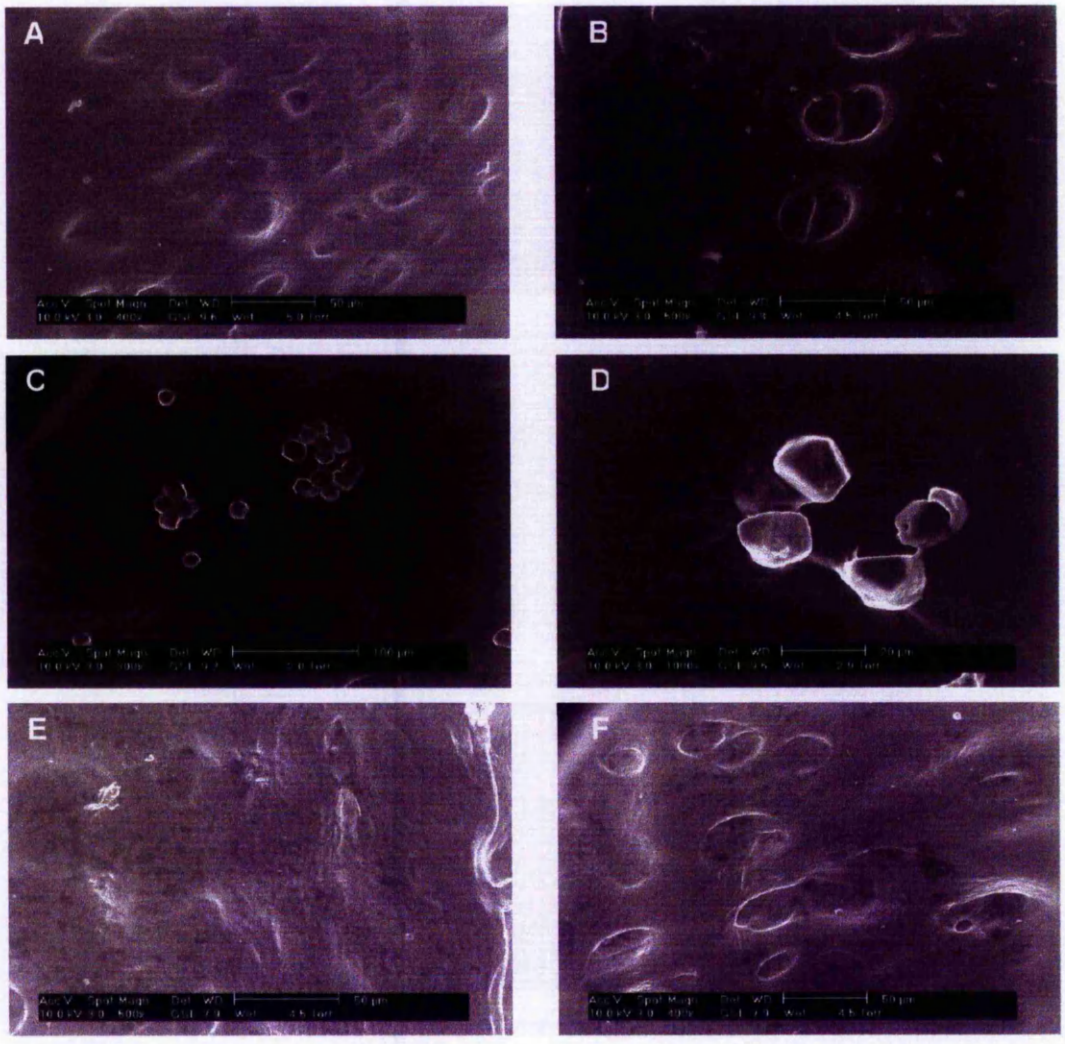


Figure IV-2.

Fig IV-2 A, B – Untreated cut cartilage surfaces viewed using ESEM, magnification x 400 and x 500 respectively

Fig IV-2 C & D – Cut cartilage surface, viewed using ESEM, that had been one side of a cartilage-cartilage interface bonded by tissue transglutaminase (4mg/ml) and physically separated before viewing, magnification x 300 and x 1000 respectively.

Fig IV-2 E & F – GAG-digested cut cartilage surfaces viewed using ESEM, magnification x 500 and x 400 respectively. GAG-digestion occurred using CABC (1U/ml) for 15min at 37°C prior to viewing (see IV-2.2).

IV-3.3 Analysis of cartilage surfaces by reflection Confocal Laser Scanning Microscopy

Using Confocal Laser Scanning Microscopy (CLSM), the surface of cartilage samples can be imaged by scanning the tissue with a laser and using the variation in the reflection of the beam as a measure of surface topography.

Figure IV-3 shows images of cut cartilage surfaces, with and without digestion of their surface GAG's, as analysed using CLSM performed on a LEICA TCS-NT microscope.

Cartilage samples subjected to GAG-digestion (using CAC, 1U/ml for 15 and 60 minutes, Fig IV-3 B/C and D/E respectively) show no apparent alterations in surface structure compared to undigested controls (Fig IV-3 A & B). The multiple circular features seen using SEM and ESEM analysis (see IV-3.1, IV-3.2) are visible once more on all the tissue surfaces, but the digestion of GAG's does not produce a visible change in surface structure as observed using this method of analysis.

The surfaces could not be imaged under liquid as this interfered with laser reflection, so the heat of the laser did cause the samples to dry out. This may have lead to artifacts within the image and means that the cartilage is not being imaged in its natural, wet state. Any differences in surface structure between the digested and undigested samples may have been lost due to this, and the same problem is encountered to varying extents when using other methods for analysing cartilage structure, such as SEM, due to the high water content of the tissue.

The smooth, glassy areas of surface that are visible in the undigested samples (Fig IV-3 A & B), but are less apparent in the digested samples, are due to surface water rather than the fact that the surface proteoglycans were not digested. These areas disappeared during imaging due to the drying effect of the laser.

Figure IV-3 Analysis of cut, hyaline, costal cartilage surfaces by Confocal Laser Scanning Microscopy in reflection mode, with and without prior digestion of glycosaminoglycan groups from the tissue surface

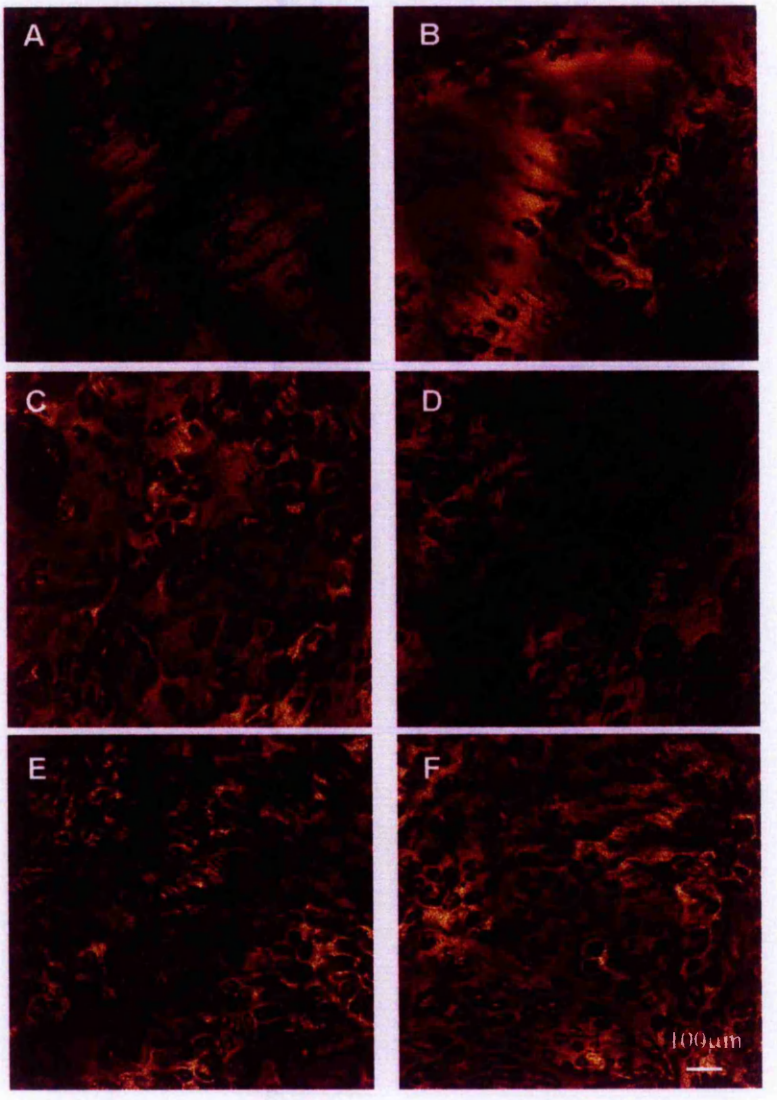


Figure IV-3.

Fig IV-3 A & B – Untreated cut cartilage surfaces viewed using CLSM, magnification x 10.

Fig IV-3 C & D – GAG-digested cut cartilage surfaces, viewed using CLSM, magnification x 10. Digestion occurred using CAC (1U/ml) for 15 min at 37°C prior to viewing (see IV-2.3)

Fig IV-3 E & F – GAG-digested cut cartilage surfaces, viewed using CLSM, magnification x 10. Digestion occurred using CAC (1U/ml) for 60 min at 37°C prior to viewing (see IV-2.3)

IV-3.4 Analysis of cartilage surfaces by Atomic Force Microscopy (AFM)

Whilst the features apparent on cut cartilage surfaces analysed by SEM, ESEM and CLSM were consistent with the published images of cartilage surfaces (albeit produced mainly from SEM and TEM analysis of articular cartilage). They failed to show any differences in surface topography between tissue samples that had been pre-treated to remove GAG's from their surface and those that remained undigested. It has also been suggested that the surface irregularities (pits, hollows, bumps etc) seen on cartilage surfaces following SEM analysis are shrinkage artifacts produced by the preparation conditions (Jurvelin *et al*, 1996). Whilst the SEM analysis performed here used frozen samples to avoid harsh preparation conditions, the samples were still therefore not in their natural state when viewed. Artifacts may have also occurred to varying extents during analysis of cartilage samples by CLSM, as the laser performing the analysis rapidly dried the tissue.

An alternative technique for surface analysis was therefore investigated, the use of Atomic Force Microscopy (AFM).

AFM uses a piezoelectric motor to move a sharp stylus tip across the surface of samples, which builds up an image of the surface and can give 3-D topographical data of the sample being examined. The advantage of AFM is that it is non-invasive and non-drying, so cartilage surfaces can be imaged under liquid and drying artifacts can be avoided. In terms of comparison of the surface before and after GAG-digestion, a further benefit of AFM is that it allows the same area of tissue to be re-imaged before and after digestion. Rather than comparing regions of different tissue samples.

AFM analysis of articular cartilage surface has shown an acellular, non-fibrous superficial layer that, upon digestion with Chondroitinase AC, reveals an underlying network of collagen fibrils (Jurvelin *et al*, 1996). The surface irregularities observed in previous SEM studies were not apparent. This fibrous substructure was also visible in certain regions of untreated surface, but was revealed with greater clarity following CAC digestion.

In this study, AFM analysis of cut cartilage surfaces did not reveal any fine structure detail that could be compared with other findings. However, the technique did provide information on surface topography, which was found to alter following GAG-digestion.

Figure IV-4 shows a region of cartilage surface, as assessed by AFM, before and after successive one minute GAG-digestions with CAC (1U/ml) at room temperature. Each 50μm scan (Fig IV-4B, IV-4C, IV-4D) represents the same area of tissue and Fig IV-4A shows a wider region of an untreated surface.

AFM analysis indicates the cut cartilage surface to be comparatively rough, at a microscopic level, before digestion of its GAG constituent (Fig IV-4A, IV-4B).

After digestion for one minute with CAC (1 U/ml), the surface appears to become less rough as the maximum peak height reduces from 3.12μm (Fig IV-4B) to 2.69μm (Fig IV-4C). This 'smoothing' of the surface appears to continue following a further CAC-digest of one minute, with the maximum peak height falling to 2.65μm (Fig IV-4D).

This effect may contribute to the increased adhesion shown by tTG at GAG-digested cartilage-cartilage interfaces, by allowing the two tissue surfaces to come into closer proximity. Thus then providing tTG with more points of direct contact between the two surfaces, with which it may cross-link the interface together and promote adhesion.

Figure IV-5 shows images of the same regions of cartilage surface as Figure IV-4, but from a birds-eye view rather than as 3-D projections.

The high level of magnification of these images means that these changes in surface topography are on a microscopic level.

Figure IV-4 Analysis of cut, hyaline, costal cartilage surfaces by Atomic Force Microscopy (AFM), with and without prior digestion of glycosaminoglycan groups from the tissue surface (using Chondroitinase AC 1U/ml for 1 or 2 minutes)

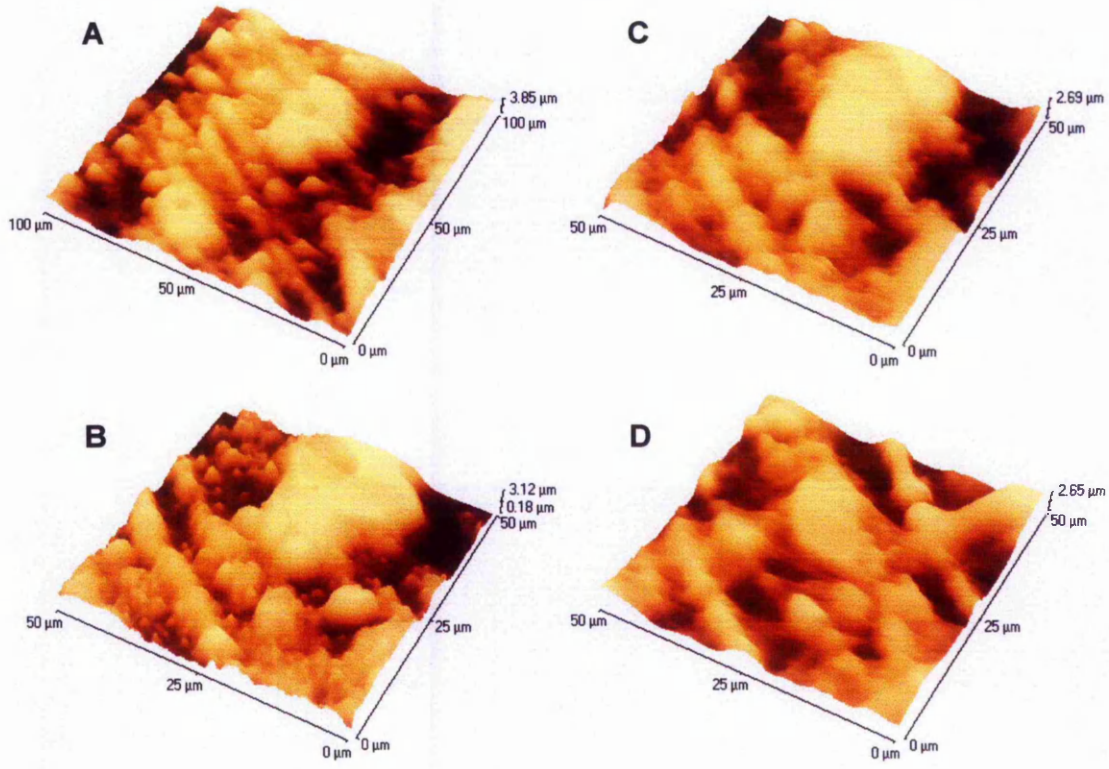


Figure IV-4.

Fig IV-4 A - Untreated cut cartilage surface viewed using AFM, 100µm scan.

Fig IV-4 B - Untreated cut cartilage surface viewed using AFM, 50µm scan.

Fig IV-4 C - Digested cut cartilage surface viewed using AFM (post 1 minute digestion with CAC (1U/ml) prior to viewing, see IV-2.4), 50µm scan.

Fig IV-4 D - Digested cut cartilage surface viewed using AFM (post 2 minutes digestion with CAC (1U/ml) prior to viewing, see IV-2.4), 50µm scan.

Figure IV-5 Analysis of cut, hyaline, costal cartilage surfaces by Atomic Force Microscopy (AFM), with and without prior digestion of glycosaminoglycan groups from the tissue surface (using Chondroitinase AC 1U/ml for 1 or 2 minutes)

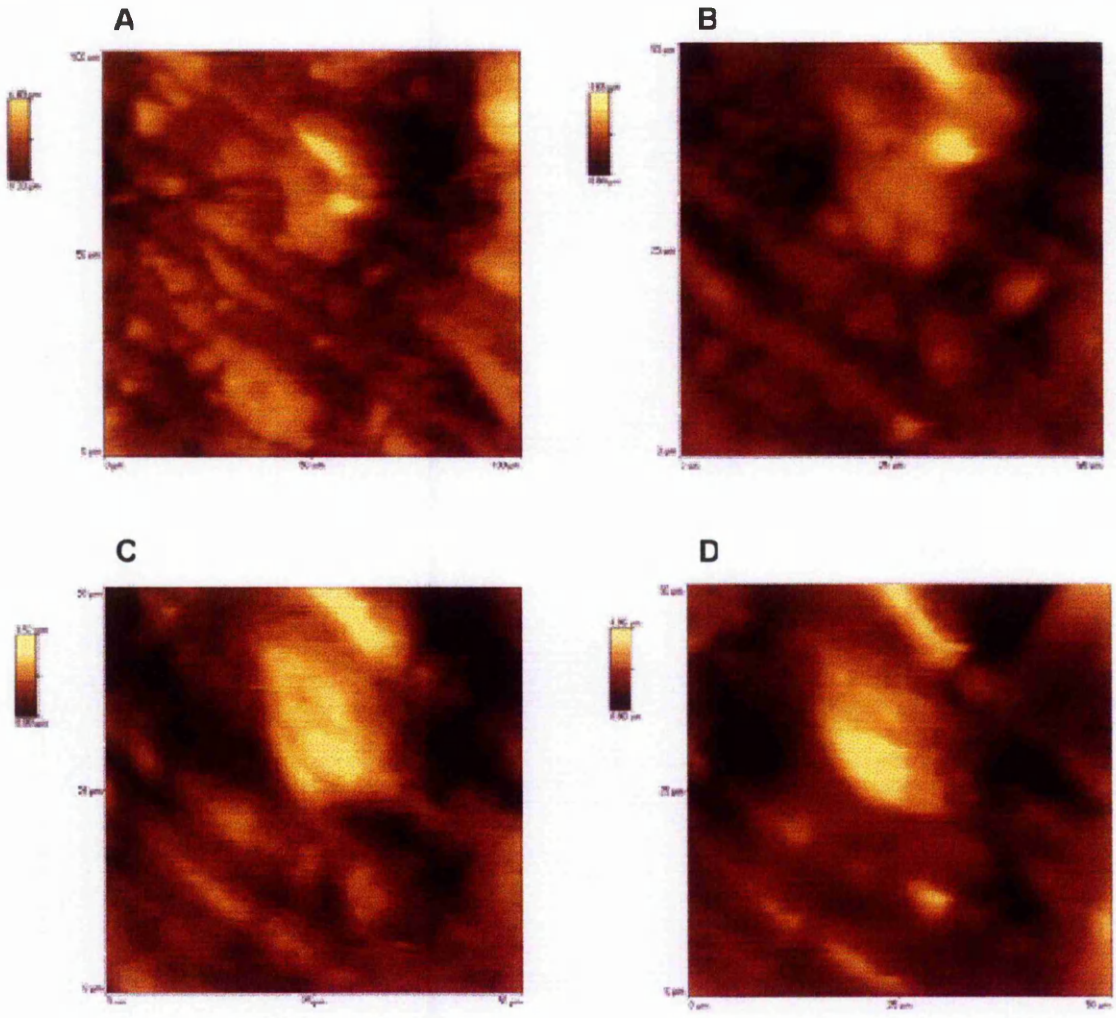


Figure IV-5.

Fig IV-5 A - Untreated cut cartilage surface viewed using AFM, 100μm scan.

Fig IV-5 B - Untreated cut cartilage surface viewed using AFM, 50μm scan.

Fig IV-5 C - Digested cut cartilage surface viewed using AFM (post 1 minute digestion with CAC (1U/ml) prior to viewing, see IV-2.4), 50μm scan.

Fig IV-5 D - Digested cut cartilage surface viewed using AFM (post 2 minutes digestion with CAC (1U/ml) prior to viewing, see IV-2.4), 50μm scan.

IV-3.5 *In situ* incorporation of fluorescent TG substrates into cut cartilage surfaces - a measure of available peptide-bound γ-glutamyl groups for tTG cross-linking

It has been proposed that GAG-digestion of a cartilage-cartilage interface may reveal increased numbers of tTG-substrate residues on the tissue surfaces (Jurgensen *et al*, 1997). These in turn may then allow for the increased bond strength shown by tTG at digested interfaces by allowing for increased levels of TG-catalysed cross-linking between the two surfaces.

The availability of tTG-substrate residues on treated and untreated cartilage surfaces was estimated by looking at the degree of tTG-catalysed incorporation of fluorescent TG-substrate molecules into the tissue.

An increased incorporation of fluorescein cadaverine (FC, fluorescent TG substrate primary amine) into a tissue surface will indicate a greater number of potential TG-substrate glutamine residues being available thereupon (since cadaverine is a competitive primary amine substrate of TG and can be incorporated into peptide-bound glutamine residues instead of peptide-bound lysine).

Figure IV-6 shows the degree of incorporation of fluorescein cadaverine into cartilage section surfaces, with and without their prior pre-treatment to remove GAG's, with and without addition of exogenous tTG (as analysed using CLSM).

Increased incorporation of FC is shown into the section to which tTG was applied (Fig IV-6B), compared to that which received no tTG (Fig IV-6A). This increase is absent when tTG is applied in the presence of tTG inhibitors such as EDTA and cystamine (Fig IV-6C, IV-6D respectively).

There is a degree of endogenous incorporation of FC into the tissue surface (Fig IV-6B), though no further studies were undertaken to determine the endogenous TG within the cartilage that may have been responsible. The exact nature of the TG's expressed in cartilage is still somewhat uncertain (see I-4.3.2).

Pre-treating the cartilage surface with CABC (1U/ml) leads to an increase in FC incorporation in a time-dependent manner with respect to the duration of the digestion (Fig IV-6E, IV-6F compared to undigested sample Fig IV-6A). This increase was also observed in the sections that received no tTG (Fig IV-6G, IV-6H endogenous incorporation), though to a much lesser extent.

Figure IV-6 *In situ* incorporation of fluorescein cadaverine (FC) into hyaline costal cartilage section surfaces, with and without prior pre-treatment of the surfaces to remove glycosaminoglycan groups.

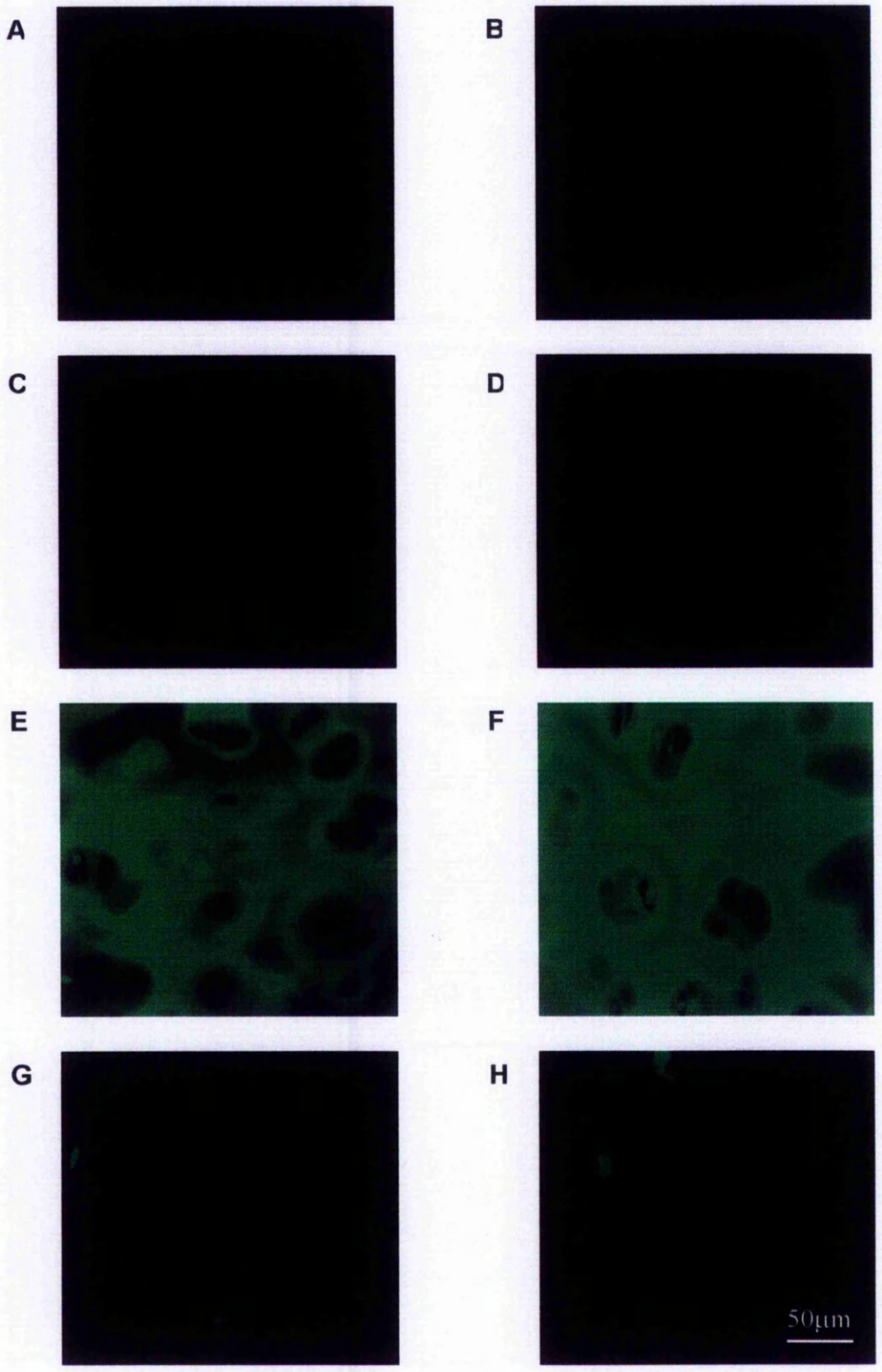


Figure IV-6.

In situ incorporation of fluorescein cadaverine (FC) into hyaline costal cartilage section surfaces, with and without prior pre-treatment of the surfaces to remove glycosaminoglycan groups.

FC incorporation into tissue sections was catalysed by exogenous applied tTG (5µl, 2mg/ml) or endogenous enzyme (see IV-2.5).

Sections were viewed using CLSM, magnification x40.

Fig IV-6 A – Endogenous incorporation of FC into an untreated cut cartilage section.

Fig IV-6 B – Exogenous tTG-catalysed incorporation of FC into an untreated cut cartilage section.

Fig IV-6 C – Exogenous tTG-catalysed incorporation of FC into an untreated cut cartilage section, in the presence of EDTA (150mM).

Fig IV-6 D – Exogenous tTG-catalysed incorporation of FC into an untreated cut cartilage section, in the presence of cystamine (60µM).

Fig IV-6 E – Exogenous tTG-catalysed incorporation of FC into a GAG-digested cut cartilage section (digestion occurred using CABC (1U/ml) for 5 minutes at 37°C).

Fig IV-6 F – Exogenous tTG-catalysed incorporation of FC into a GAG-digested cut cartilage section (digestion occurred using CABC (1U/ml) for 15 minutes at 37°C).

Fig IV-6 G – Endogenous incorporation of FC into a GAG-digested cut cartilage section (digestion occurred using CABC (1U/ml) for 5 minutes at 37°C).

Fig IV-6 H – Endogenous incorporation of FC into a GAG-digested cut cartilage section (digestion occurred using CABC (1U/ml) for 15 minutes at 37°C).

Digestion of GAG's from a cartilage surface therefore reveals more potential TG-substrate glutamine residues thereupon, which allow for the increased levels of tTG-catalysed incorporation of fluorescein cadaverine into the tissue.

The greater bond strength shown by tTG at a GAG-digested cartilage-cartilage interface (compared to an untreated one) therefore may also be due to these biochemical alterations in the tissue surface.

As well as removing negative charge from the cartilage (reducing its anti-adhesive properties), GAG-digestion lowers the microscopic surface topography of the interface (as found from AFM analysis) and exposes increased numbers of peptide-bound γ-glutamyl residues. These may then allow for increased levels of tTG-catalysed bonding between the cartilage surfaces, by increasing the number of points of direct contact between the interface and by increasing the number of potential tTG-substrate, peptide-bound γ-glutamyl residues exposed on the tissue surfaces.

IV-4 Conclusions

Various surface analysis techniques were used to study the topography and biochemistry of cut, hyaline, costal-cartilage surfaces, before and after the surfaces were subjected to enzymatic digestion of their glycosaminoglycan constituent.

Scanning Electron Microscopy, Environmental Scanning Electron Microscopy and Confocal Laser Scanning Microscopy profiling all produced images of cut cartilage surfaces, whose features broadly corresponded to those images of cartilage surfaces found in the literature. These published images mainly came from SEM analysis of articular cartilage surfaces, but the same multiple undulations and circular features were seen in all the surfaces.

However, using SEM, ESEM and CLSM, no differences in surface structure were observed between untreated cartilage surfaces and those digested to remove GAG's from the tissue surface.

In most electron microscopy sections, large GAG arrays generally collapse to small dense granules as their hydration sheath is stripped away during sample preparation. But when prepared by cryo-technology, their complex nature should still be visible (Hunziker & Schenk, 1984). The SEM analysis performed here used cryo-technology to preserve the cartilage sample during viewing and indeed, structures possibly attributable to proteoglycan/glycosaminoglycan complexes were visible at magnifications of x1000 and greater. However, the number and size of these complexes did not appear to differ between an untreated surface and one subjected to a 15-minute digest of its GAG's by Chondroitinase AC.

There is a growing body of evidence to suggest that the surface irregularities (pits, hollows, bumps etc), observed by SEM analysis of cartilage surfaces, are shrinkage artifacts produced by the de-hydrating preparation conditions (Jurvelin *et al*, 1996). SEM analysis has often contradicted the findings of TEM studies of articular cartilage surfaces, which report a smooth surface without the many irregularities highlighted by SEM techniques. Where care is taken to preserve hydration of the tissue during SEM analysis, a smooth articular surface is also found (Bloebaum & Wilson, 1980).

Whilst the SEM analysis performed here attempted to use cryo-technology in place of harsh dehydrating preparation techniques, the tissue was still not being imaged in its natural state and so surface features seen may be attributable to artifacts.

The same also applies for the CLSM analysis performed, whose laser profiling dried out the cartilage samples during viewing.

A further investigation of cut cartilage surface structure was then carried out using Atomic Force Microscopy (AFM).

The advantage of AFM analysis is that it allows cartilage to be imaged under liquid, thus retaining its water content and structural integrity. It also allows the same area of tissue to be re-imaged before and after GAG-digestion, rather than comparing digested and undigested regions of separate samples. The ESEM analysis performed did allow tissue samples to remain hydrated whilst viewing, but the same area of sample could not be viewed before and after GAG-digestion. So any comparisons made were between different areas of tissue.

AFM analysis showed the cut cartilage surface to comparatively rough before digestion of its GAG constituent (Fig IV-4A, IV-4B). Digestion of the tissue surface with CAC (1 U/ml), then smoothed out the microscopic surface topography over time (Fig IV-4C, IV-4D).

This effect may contribute to the increased adhesion shown by tTG at GAG-digested cartilage-cartilage interfaces, by allowing the two tissue surfaces to come into closer proximity. Providing tTG with more points of direct contact between the two surfaces, with which it may cross-link the interface together and promote adhesion.

AFM failed to produce any fine detail of the cut cartilage surfaces, though it is often difficult to identify the nature of individual components of imaged structures using AFM analysis of biological specimens (Lal & John, 1994).

Alongside the analysis of the topography of cut cartilage surfaces, their biochemistry was also investigated in terms of the degree of tTG-catalysed incorporation of fluorescent TG-substrate molecules into the tissue surface.

An increased incorporation of fluorescein cadaverine (fluorescent primary amine TG substrate) into a tissue surface indicates the presence of a greater number of exposed peptide-bound γ-glutamyl residues being available for tTG-catalysed cross-linking.

Treating the cut cartilage surface with Chondroitinase ABC led to an increase in tTG-catalysed FC incorporation in a time-dependent manner with respect to the duration of the digestion.

Digestion of GAG's from a cartilage surface therefore reveals more potential peptide-bound TG-substrate glutamine residues thereupon, which allow for the increased levels of tTG-catalysed incorporation of fluorescein cadaverine into the tissue.

The greater bond strength shown by tTG at a GAG-digested cartilage-cartilage interface (compared to an untreated one) may therefore be due to a variety of alterations occurring upon the tissue surfaces.

As well as removing negative charge from the tissue surfaces and reducing their anti-adhesive properties, GAG-digestion smoothes out the microscopic surface topography of the interface allowing increased points of contact. It also exposes increased numbers of peptide-bound γ-glutamyl residues on the tissue surface, allowing for increased levels of tTG-catalysed bonding between the cartilage-cartilage interface and hence, greater bond strength.

This finding also means that cartilage repair materials and implants can be tailored in order to maximise tTG-catalysed bonding at the interface between biomaterials and tissue, promoting adhesion and retention of the implant and helping to promote tissue repair. Increasing the number of ε-lysine groups available on a biomaterial, and digesting the cartilage surface intended for bonding to remove GAG's, allows for increased levels of tTG-catalysed cross-linking between the tissue-biomaterial interface. These increased numbers of cross-links are formed between the revealed γ-glutamyl groups on the cartilage and the ε-lysine groups upon the biomaterial, and should then lead to increased bond strength at the tissue-biomaterial interface

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Chapter V : Additional bond strength studies - Additional proteins and polyamines, alternative Transglutaminases and the bonding of biomaterials to cartilage surfaces

V-1 Introduction

Adhesive preparations investigated thus far have comprised tissue transglutaminase enzyme alone, along with an activating buffer. Studies were also undertaken to determine the bond strength of tTG preparations containing additional tTG-substrate proteins and polyamines, as it was thought that their inclusion might raise the bonding strength of the preparation by acting as structural bridges. As such, they would be cross-linked by tTG to the cartilage surfaces and help to bridge any gaps that may occur along the tissue interface (a theory also put forward by Jurgensen *et al*, 1997). The bond strength of tTG preparations containing TG-substrate polyamines and proteins such as fibronectin, collagen, casein and albumin were all determined at cartilage-cartilage interfaces using the novel *in vitro* test method developed.

Also investigated was the effect of adding a thickening agent to the tTG adhesive. The solutions all used thus far have been very fluid, which may become a problem for the surgical user in retaining the adhesive at the site of application. Therefore the polysaccharide pullulan was added to thicken the adhesive and the effects of this on bonding strength were measured. Pulluan is a naturally occurring starch-like substance produced by *Aureobasidium pullulans*, that has also been proposed as an additive in the food industry for texturising meat (Kimoto *et al*, 1997).

After showing that GAG-digestion of cartilage surfaces increases the number of exposed γ-glutamyl residues available for cross-linking by tTG (see ChIV), lysine-rich proteins and polyamines were added to tTG preparations applied to digested interfaces to see if they could raise bonding strength. The idea being to provide numerous lysine residues for tTG to cross-link with the increased numbers of exposed γ-glutamyl residues, raising the amount of cross-linking between the interface surfaces and promoting bonding. Proteins investigated in this way included poly-l-lysine and bi-functional polyamines such as putrescine were also studied.

Knowing that digestion of GAG's leads to the exposure of peptide-bound γ-glutamyl groups on the cartilage surface also facilitates the optimal design of any biomaterials that are to be bonded to the tissue by transglutaminase.

Many cartilage repair strategies involve the implantation of biomaterials and matrices into cartilage defect sites. Not only can these be now optimised for their primary purpose (e.g. to act as a scaffold for cell re-population of a defect), but they can also be selected for their retention at the defect site via TG-catalysed bonding.

Biomaterials designed for cartilage repair may be tailored so that they may contain increased numbers of ε-lysine residues to take advantage of the increased numbers of exposed γ-glutamine residues present on GAG-digested cartilage surfaces. This would give tTG more potential sites for catalysing cross-linking between the tissue and biomaterial, promoting adhesion of the repair material to the defect site.

In order to achieve this result the tTG-catalysed bonding of optimised biomaterials to a GAG-digested cartilage surface was also studied. In this case the optimised biomaterials selected included a synthetic polymer of poly-lactic acid-co-lysine and a biocomposite of poly(ε-caprolactone), collagen (I) and fibronectin.

As well as evaluating the performance of tTG-based adhesives, the bond strengths of alternative transglutaminase preparations were investigated at cartilage-cartilage interfaces.

Microbial transglutaminases (mTG) already have wide commercial use in the food industry, where they are used to bind food protein (Yasuyuki, 1996). Their relative ease of large-scale, cheap production is one attractive feature that has led to their proposal in biomedical applications (see I-4.4). A TG derived from micro-organisms has already been proposed as the active component in both a wound protecting composition and living tissue adhesive (Kitahara *et al*, 1996; Mawatari *et al*, 1995).

The mTG used in this study is Ajinomoto transglutaminase, derived from the culture filtrate of the strain S-8112, supposed to belong to the genus *Streptovercillium* (Ando *et al*, 1989). It was found to consist of a single polypeptide chain of Mr ~38,000, which is around half the molecular weight of a TG derived from guinea-pig liver (used as a comparison during characterisation of this microbial TG). It contains 331 amino acids, has no Ca^{2+} binding site within its structure and is a calcium independent

enzyme, unlike tTG (as stated in the product literature). When its activity was measured using synthetic substrates, the enzyme showed high activity over a wide pH range (pH 5-8). Whilst the enzyme did not utilise some of the synthetic substrates used (unlike the guinea-pig liver TG), its substrate specificity towards other synthetic peptides was lower than that of the mammalian enzyme (Ando *et al*, 1989). Food proteins shown to be good substrates for this microbial TG include casein, gelatin, collagen and myosin. As well as soy and wheat proteins, which contain many lysine and glutamine residues. The enzyme is also stable at temperatures up to 40°C, with activity decreasing gradually after about 50°C. Thus there are various differences between the microbial TG and that derived from guinea-pig liver, in terms of thermal stability, molecular weight, calcium requirement and substrate specificity.

The Ajinomoto enzyme was supplied in two preparations. One, termed 'Activa-EB', is a meat-binding product containing 0-5% microbial TG, 39.5% maltodextrin and 60.0% caesinate. The second, termed 'Activa KHM2', consisted of 10% microbial TG and 90% maltodextrin. The bonding strengths of both these preparations were investigated as found and also after attempts to purify the mTG from the ingredients supplied with the enzyme.

Another transglutaminase studied was the plasma TG Factor XIII. This took the form of a commercial healthcare product 'Fibrogammin®' (Centeon, UK) supplied as a vial of 250 U of Factor XIII (along with human-albumin, glucose and sodium chloride) plus water for reconstitution. The product is administered to patients with blood-clotting deficiencies, to supplement the action of the Factor XIII in their circulation. As the activation-state of the Factor XIII was unknown, its bond strength was determined at cartilage-cartilage interfaces in the presence of both calcium and thrombin.

V-2 Methods

V-2.1 Measurement of tissue transglutaminase bond strength at cartilage-cartilage interfaces in the presence of additional proteins and polyamines

Tissue transglutaminase was prepared as before (see III-2.1) and measurement of tTG bond strength was as previous (see III-2.1.1). Results show mean bond strength (MPa) ± standard error, with four samples tested per condition (unless otherwise stated).

Additional proteins or polyamines were re-constituted in 50mM Tris.Cl buffer (pH 7.3) and combined with tTG solution immediately prior to application to the cartilage-cartilage interface. Concentrations given (for tTG also) are with respect to the 5µl of tTG/protein solution applied. 5µl of Tris activating buffer (TAB, see III-2.1.1) was also applied to the opposing surface of the interface as standard.

Polyamines used were putrescine di-hydrochloride, spermidine tri-hydrochloride and spermine tetra-hydrochloride. Proteins used included gelatin (Type A (175 bloom) from porcine skin re-constituted and stored at 37°C to prevent gelation), pullulan, fibronectin (1mg/ml solution), collagen (acid-soluble Type II from bovine tracheal cartilage re-constituted in 0.1M acetic acid), bovine albumin, caesin and poly-l-lysine hydrobromdide.

The bond strength of tTG in the presence of additional proteins was also investigated at cartilage-cartilage interfaces treated to digest the GAG-constituent from the tissue surfaces. GAG-digestion occurred as previous (see III-2.3.4).

V-2.2 Bond strength of alternative transglutaminases at cartilage-cartilage interfaces

V-2.2.1 Microbial transglutaminase

The bond strength of transglutaminase derived from micro-organisms (microbial TG, mTG) was investigated using two commercial preparations.

The mTG used was produced by Ajinomoto Co (Japan) and derived from the culture filtrate of the strain S-8112, supposed to belong to the genus *Streptoverticillium*.

V-2.2.1.1 Activa-EB

'Activa-EB' (Ajinomoto Co, Japan) is a meat-binding product containing 0-5% microbial TG, 39.5% maltodextrin and 60.0% caesinate. The bond strength of Activa-EB was measured using the novel method described (see II-2) and three Activa-EB preparations were tested:

1. Activa-EB re-constituted according to manufacturers recommended re-hydration ration (6-10g AEB + 20-30g H_20) and 10µl of this solution applied to one surface of the cartilage-cartilage interface
2. Activa-EB powder applied directly to cover one surface of the cartilage-cartilage interface
3. Activa-EB powder applied directly to cover one surface of the cartilage-cartilage interface and 10µl of Tris-buffered saline (pH 7.3) applied to the other.

For all, the tissue surfaces were then re-opposed and incubation occurred as standard.

V-2.2.1.2 'Activa KHM2'

'Activa KHM2' (Ajinomoto Co, Japan) consists of 90% maltodextrin and 10% microbial TG. The preparation was re-constituted in 50mM Tris.Cl buffer (pH 6.5) to a concentration of 10mg/ml (total w/v, therefore a 1mg/ml mTG content assuming a proportion of 90% maltodextrin, 10% TG).

This was assayed for TG activity via radio-labelled putrescine incorporation into N,N'-dimethylcaesin (see II-3) at pH 6.5 using both 1 and 5mg/ml (total w/v) solutions.

KHM2 solution was also analysed via SDS-PAGE on a 10% acrylamide content resolving gel (see II-5). 10µl of KHM2 solution was run per lane using both 50 and 5mg/ml solutions (total w/v, therefore 5 and 0.5 mg/ml mTG content).

The protein content of a 10mg/ml KHM2 solution (total w/v) was estimated using the BIO-RAD Lowry method (see II-4).

The bond strength of KHM2 at a cartilage-cartilage interface was measured using the novel method described (see II-2). 5µl of KHM2 solution (20, 40 and 80mg/ml total w/v in 50mM Tris.Cl buffer (pH 6.5), therefore 2, 4 and 8 mg/ml mTG content) was applied to one interface surface and 5µl of 50mM Tris.Cl buffer (pH 6.5) was applied to the other. Incubation and testing occurred as standard (see II-2).

In an attempt to remove the maltodextrin from the KHM2 preparation, it was subjected to ammonium sulphate precipitation.

51.6g of ammonium-sulphate was added to a 100ml of KHM2 solution (3mg/ml total w/v, 0.3mg/ml mTG content) over 1hr at 4°C with stirring. This gave an 80% (w/v) ammonium-sulphate solution.

The solution was divided into 4 x 25ml samples and centrifuged at 10,000g for 30min at 4°C. Supernatant was then removed and each pellet re-suspended in 2mls of 50mM Tris.Cl buffer (pH 6.5). Each sample was assayed for protein content via the BIO-RAD Lowry assay (see II-4).

One sample ('A') was assayed for activity via radio-labelled putrescine incorporation into N,N'-dimethylcaesin (see II-3).

The bond strength of sample 'A' was measured at a cartilage-cartilage interface using the novel method described (see II-2). 5µl of sample was applied to one interface surface and 5µl of 50mM Tris.Cl buffer (pH 6.5) to the other. Incubation and testing occurred as standard.

V-2.2.2 Factor XIII

The bond strength of a preparation containing the plasma transglutaminase, Factor XIII was investigated using Fibrogammin® (Centeon, UK) a commercial healthcare product supplied in two vials:

1. 68-135 mg of solid (24-64 mg protein) containing 250 U of Factor XIII [1 unit being equivalent to the factor-XIII-activity of 1ml of fresh citrated plasma (pooled plasma) of healthy donors]. Also present are: 24-40 mg human-albumin, 16-24 mg glucose and 28-44 mg sodium chloride.
2. 4ml of H_20 for injection.

The contents of vial 1 were re-constituted with those of vial 2 to give a solution of 62.5 U/ml that was stable for 8 hrs whilst stored at 2-8°C (as stated in product literature). Factor XIII solution was assayed for activity via radio-labelled putrescine incorporation into N,N'-dimethylcaesin (see II-3) and protein content via the BIO-RAD Lowry assay (see II-4).

Measurement of Fibrogammin® (Factor XIII) bond strength was performed using the novel method developed (see II-2).

Since the product literature contained no mention of the activation-state of the Factor XIII in the Fibrogammin® solution, its bond strength was measured at the tissue interface using:

1. 5µl Fibrogammin + 5µl TAB (for direct comparison with tTG). Assuming the Factor XIII was present as FXIIIa and therefore needed only calcium for its activation.
2. 5µl Fibrogammin + 5µl 40mM calcium chloride solution (in 50mM Tris.Cl pH 7.3) containing 250 U/ml of thrombin. Assuming the FXIII required the proteolytic action of thrombin plus calcium for its activation.

V-2.3 Bond strength of tTG at cartilage-synthetic polymer interfaces

The bond strength of tTG at cartilage-synthetic polymer interfaces was investigated using the novel test method outlined (see II-2).

The polymers used were poly (lactic acid-co-lysine) and a biocomposite of poly(ε-caprolactone), fibronectin and collagen I, which were both cast as thin film discs by Robert Saint (Nottingham Trent University).

Poly (lactic acid-co-lysine) was prepared using the method of Barrera *et al* (1993) and NMR studies showed the lysine content to be 2.34% with 50% deprotection, i.e. 50% of the lysines were available for cross-linking.

The biocomposite used the method of Coombes *et al* (2000), with the collagen and fibronectin present at a ratio of 12.5 to 1.

After preparing a tissue sample to create a cartilage-cartilage interface (see II-2.1), each of the two cartilage surfaces received 5µl of tTG solution and 5µl of TAB. A polymer disk pre-soaked in 50mM Tris.Cl buffer (pH 7.3) was then placed between the two opposing cartilage surfaces. Each of the two polymer-cartilage interfaces formed had therefore received 5µl of tTG solution and 5µl of TAB, the same as for a standard cartilage-cartilage interface. Incubation and testing then occurred as standard.

V-2.3 Statistics

The data relating to bond strength was compared by two-way analysis using the students' t-test (assuming unequal variance). Significant differences ($p<0.05$) versus controls are labelled as *.

V-3 Results

V-3.1 Bond strength of tissue transglutaminase at cartilage-cartilage interfaces, in the presence of additional proteins and polyamines

V-3.1.1 Presence of a thickening agent

An essential feature of tissue adhesives is that they should be in the flow-state during application, allowing them to spread over the entire tissue surface that is to be adhered and result in effective and close contact between the adhesive molecules and tissue surfaces (Ikada, 1997). The tTG solutions used so far were all very fluid, which may actually cause a problem to the surgical user when trying to confine the adhesive to the intended surface. Therefore a thickening agent, commonly used in dermatological lotions, was added to the tTG preparation to see if this affected bonding strength.

It was found that the bond strength of tTG at a cartilage-cartilage interface was unaffected by the presence of the natural polysaccharide pullulan at both 50 and 100 mg/ml (Figure V-1).

Bond strengths achieved after incubation under standard conditions were 0.056 ± 0.008 MPa (4mg/ml tTG), 0.059 ± 0.005 MPa (4mg/ml tTG + 50mg/ml pullulan) and 0.056 ± 0.006 MPa (4mg/ml tTG + 100mg/ml pullulan).

Therefore, pullulan may be used to thicken a tTG adhesive preparation without impairing the bonding strength.

Figure V-1 Bond strength achieved at a cartilage-cartilage interface with use of tTG, with and without the presence of a thickening agent, under standard incubation conditions

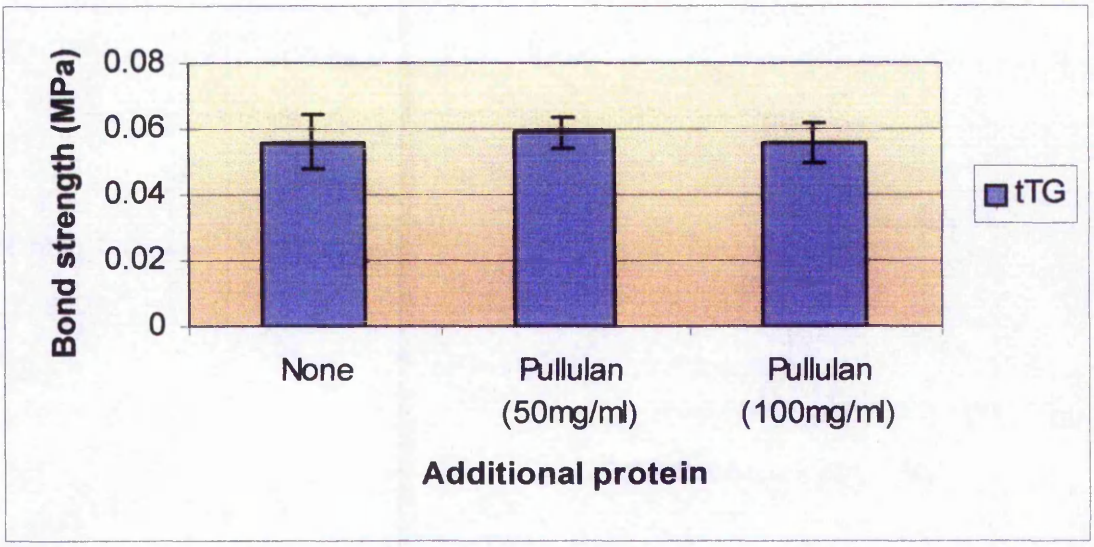

Figure V-1.

Bond strength of tTG (4mg/ml, 3084 nmol/hr/mg specific activity) ± pullulan (50 & 100mg/ml) after incubation for 20min at 37°C, 20% humidity (for method, see V-2.1). Figures show mean bond strength ± standard error (n=4).

V-3.1.2 Presence of tTG-substrate proteins and polyamines

The large substrate specificity of tTG is a feature that has led to its proposal as a tissue-bonding agent. It was thought that the bond strength of a tTG preparation might be increased by the inclusion of tTG-substrate molecules, which may serve to act as 'bridging molecules' for cross-linking by tTG, thus helping to span any gaps that occur along the tissue interface.

V-3.1.2.1 Polyamines

The bond strengths produced by tTG preparations at untreated cartilage-cartilage interfaces were compared to those shown by tTG solutions containing putrescine, spermidine and spermine, polyamine substrates for tTG.

The presence of these polyamines had no positive effect in enhancing the bond strength of a tTG preparation (Figure V-2). The bond strength of 0.054 ± 0.004MPa, for a tTG preparation (4mg/ml) under standard incubation conditions, was not significantly increased by the addition of putrescine, spermidine or spermine at concentrations of both 10mM and 50mM.

When applied to a GAG-digested cartilage-cartilage interface (CAC 1U/ml, 15 minutes prior to bonding), the presence of putrescine again did not significantly enhance the bond strength shown by a tTG preparation. tTG (4mg/ml) showed a bond strength of 0.104 ± 0.014 MPa at a GAG-digested interface (after incubation under standard conditions), compared to 0.090 ± 0.019 MPa when applied to equivalent interfaces in the presence of putrescine (50mM).

Figure V-2 Bond strength achieved at untreated and GAG-digested cartilage-cartilage interfaces with use of tTG, with and without the presence of polyamines, under standard incubation conditions

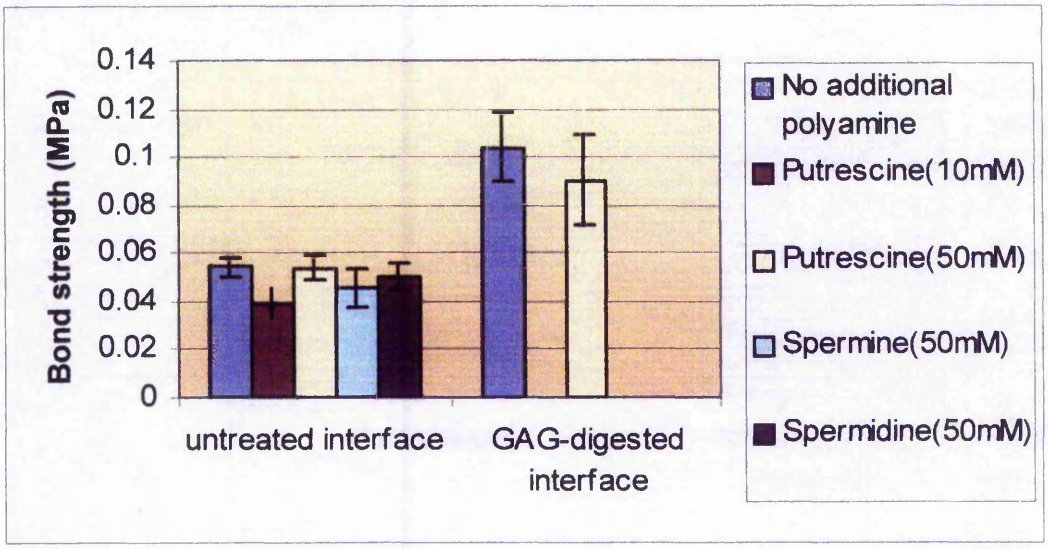

Figure V-2.

Bond strength of tTG (4mg/ml, 3522 nmol/hr/mg specific activity) ± putrescine, spermine and spermidine (either 10 or 50mM) at untreated and GAG-digested cartilage-cartilage interfaces after incubation for 20min at 37°C, 20% humidity. Interface was either untreated or digested with CAC (1U/ml) for 15 minutes prior to bonding (for method, see V-2.1). Figures show mean bond strength ± standard error (n=4).

V-3.1.2.2 Gelatin

Gelatin is both a tTG substrate and a gel-forming protein when in solution. Its inclusion in a tTG preparation led to a significant enhancement ($p<0.05$) in the bond strength produced by the preparation at a cartilage-cartilage interface.

Figure V-3 shows that bond strength rose from 0.054 ± 0.004 MPa for a tTG solution (4mg/ml), to 0.075 ± 0.006 MPa for a tTG solution (4mg/ml) containing gelatin (50mg/ml, after incubation under standard conditions).

A similar significant increase was not observed in the buffer control, which suggests that the beneficial effect of gelatin (when used with tTG) was due to it being a tTG substrate, not its ability to form gels in solution at room temperature. Since testing was performed at room temperature, any positive effect gelling may have had on the bond strength should have also been observed in the control solutions and this was not the case.

V-3.1.2.3 Albumin

Inclusion of bovine serum albumin in the tTG preparation had no beneficial effect in raising the bond strength shown at a cartilage-cartilage interface. Figure V-4 shows that tTG (4mg/ml) produced a bond strength of 0.043 ± 0.001 MPa under standard incubation conditions and 0.045 ± 0.005 MPa when applied in the presence of albumin (50mg/ml).

Figure V-3 Bond strength achieved at a cartilage-cartilage interface with use of tTG and buffer control, with and without the presence of Gelatin, under standard incubation conditions.

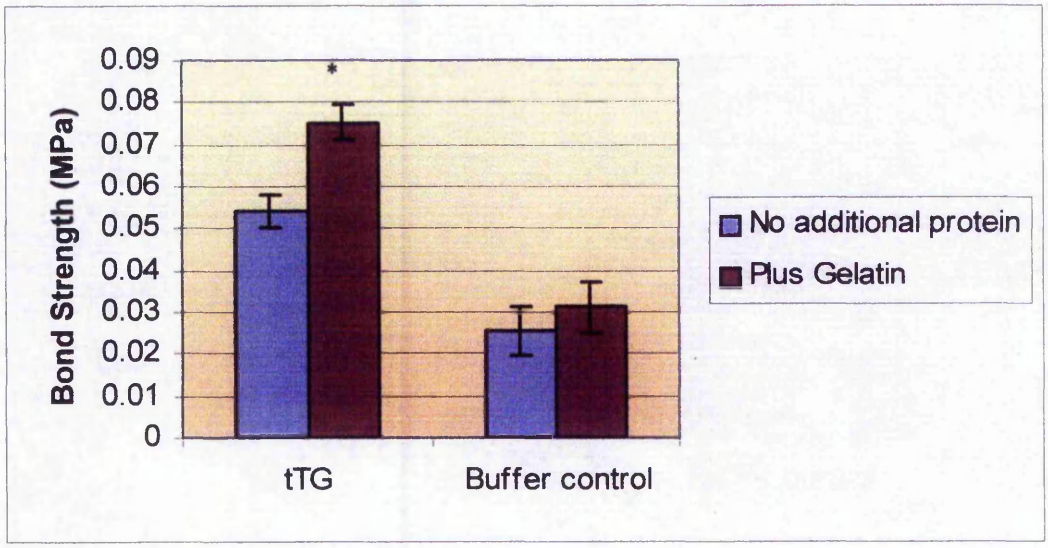

Figure V-3.

Bond strength of tTG (4mg/ml, 3522 nmol/hr/mg specific activity) and buffer control, ± gelatin (50mg/ml) after incubation for 20min at 37°C, 20% humidity (for method, see V-2.1). Figures show mean bond strength ± standard error (n=4). Statistically significant data (compared to no additional protein within each group) are marked as * ($p<0.05$).

Figure V-4 Bond strength achieved at a cartilage-cartilage interface with use of tTG with and without the presence of bovine albumin, under standard incubation conditions.

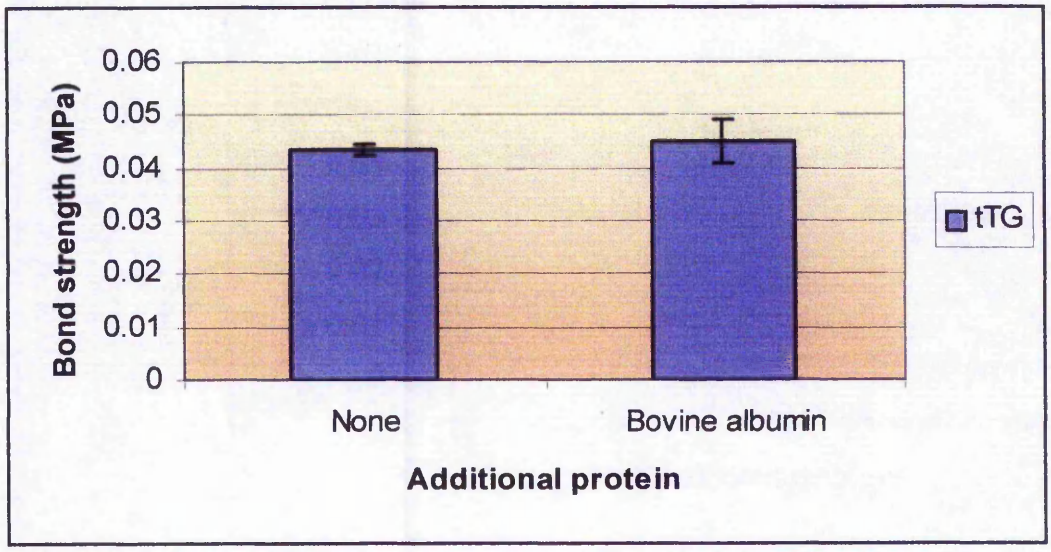

Figure V-4.

Bond strength of tTG (4mg/ml, 3064 nmol/hr/mg specific activity) ± bovine albumin (50mg/ml) after incubation for 20min at 37°C, 20% humidity (for method, see V-2.1). Figures show mean bond strength ± standard error (n=3).

V-3.1.2.4 Casein

Inclusion of casein in the tTG preparation also had no beneficial effect in raising the bond strength shown at both an untreated cartilage-cartilage interface and one whose surface had been digested to remove GAG's.

Figure V-5 shows that when applied to an untreated cartilage-cartilage interface, tTG (4mg/ml) produced a bond strength of 0.060 ± 0.018 MPa under standard incubation conditions and 0.058 ± 0.009 MPa when applied in the presence of casein (50mg/ml). When applied to an interface pre-treated with CABC prior to bonding (1U/ml for 5min), tTG (4mg/ml) in the presence of casein (50mg/ml) produced a bond strength of 0.051±0.006 MPa.

V-3.1.2.5 Poly-l-lysine

The bond strength of tTG was not increased by the presence of poly-l-lysine, when applied to both untreated and GAG-digested cartilage-cartilage interfaces.

Figure V-6 shows that tissue transglutaminase (4mg/ml) produced a bond strength of 0.043 ± 0.008 MPa when applied to an untreated interface under standard incubation conditions, compared to 0.030 ± 0.004 MPa when applied in the presence of poly-l-lysine (25mg/ml). At a GAG-digested interface (CAC, 1U/ml, 15min), tTG (4mg/ml) showed a bond strength of 0.104 ± 0.014 MPa, compared to 0.072 ± 0.008 MPa when applied on the presence of poly-l-lysine (25mg/ml).

Figure V-5 Bond strength achieved at untreated and GAG-digested cartilage-cartilage interfaces with use of tTG, with and without the presence of casein, under standard incubation conditions

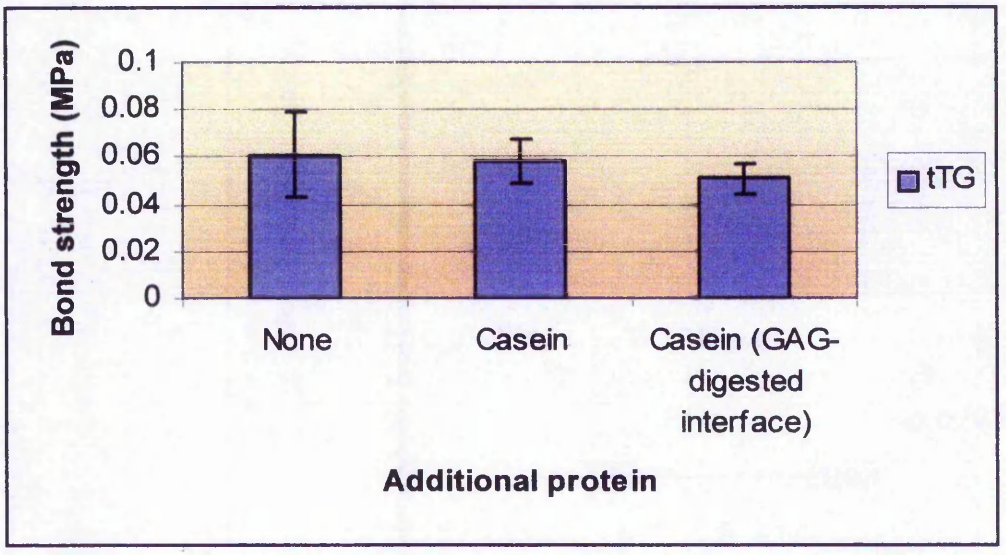

Figure V-5.

Bond strength of tTG (4mg/ml, 2888 nmol/hr/mg specific activity) ± casein (50mg/ml) after incubation for 20min at 37°C, 20% humidity. Interface was either untreated or digested with CABC (1U/ml) for 5 minutes prior to bonding (for method, see V-2.1). Figures show mean bond strength ± standard error (n=4).

Figure V-6 Bond strength achieved at untreated and GAG-digested cartilage-cartilage interfaces with use of tTG, with and without the presence of poly-l-lysine, under standard incubation conditions

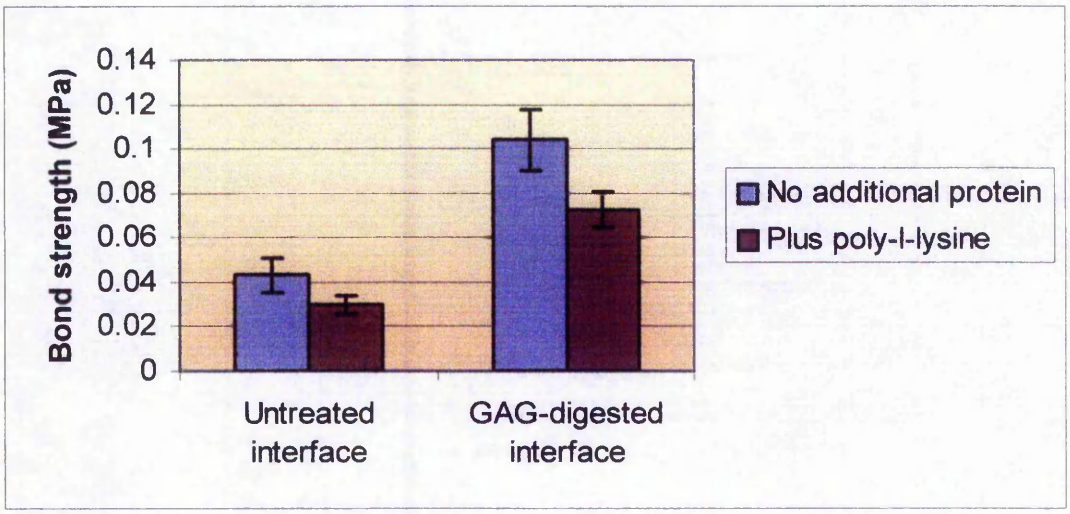

Figure V-6.

Bond strength of tTG (4mg/ml, 3185 nmol/hr/mg specific activity) ± poly-l-lysine (25mg/ml) after incubation for 20min at 37°C, 20% humidity. Interface was either untreated or digested with CAC (1U/ml) for 15 minutes prior to bonding (for method, see V-2.1). Figures show mean bond strength ± standard error (n=4).

V-3.1.2.6 Collagen and fibronectin

Collagen (II) is the main structural component of cartilage and fibronectin a constituent of its extra-cellular matrix (see I-2.1).

Figure V-7 shows that at the concentration used, the presence of fibronectin had no beneficial effect on raising the bond strength of a tTG preparation at a cartilage-cartilage interface. tTG (4mg/ml) produced a bond strength of 0.056 ± 0.008 MPa under standard incubation conditions, compared to 0.056 ± 0.001 for tTG (4mg/ml) in the presence of fibronectin (1mg/ml).

The presence of collagen (II) did raise the bond strength shown by tTG at a cartilage-cartilage interface, although the increase was not significant (Fig V-8). A bond-strength of 0.036 ± 0.001 MPa was found for tTG (4mg/ml) under standard incubation conditions. This increased to 0.063 ± 0.012 MPa in the presence of collagen (II) (2.5 mg/ml in acetic acid), despite the possible alteration of the pH of the tTG preparation by the acetic acid in which the collagen was re-constituted.

Figure V-7 Bond strength achieved at a cartilage-cartilage interface with use of tTG with and without the presence of fibronectin, under standard incubation conditions

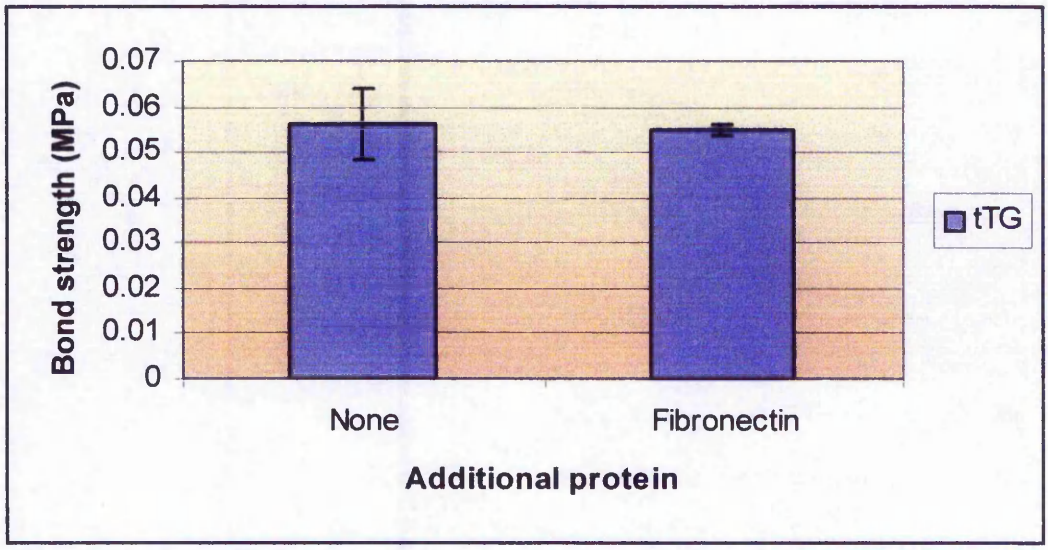

Figure V-7.

Bond strength of tTG (4mg/ml, 3084 nmol/hr/mg specific activity) ± fibronectin (1mg/ml) after incubation for 20min at 37°C, 20% humidity (for method, see V-2.1). Figures show mean bond strength ± standard error (n=4).

Figure V-8 Bond strength achieved at a cartilage-cartilage interface with use of tTG with and without the presence of collagen (II), under standard incubation conditions

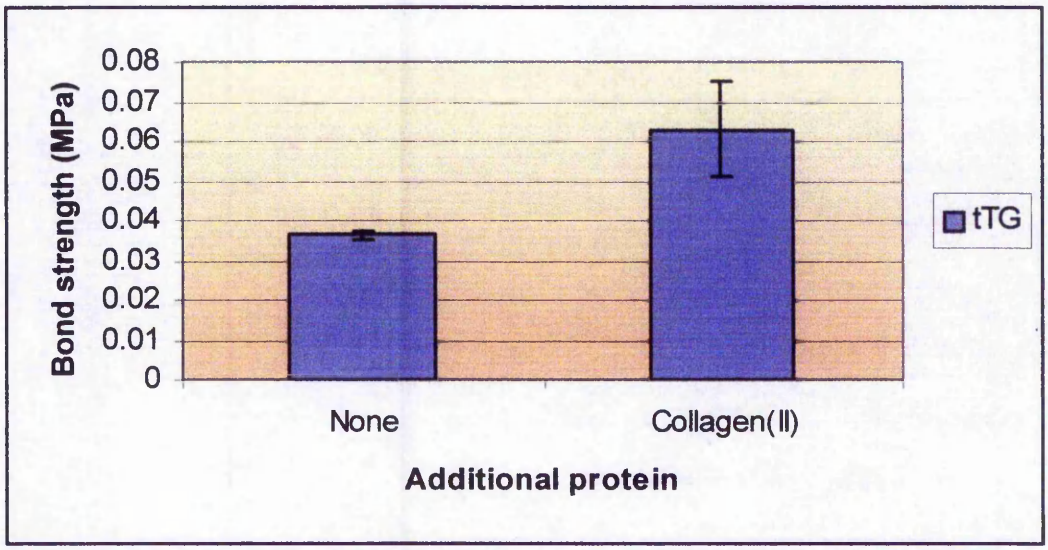

Figure V-8.

Bond strength of tTG (4mg/ml, 3184 nmol/hr/mg specific activity) ± collagen (2.5mg/ml) after incubation for 20min at 37°C, 20% humidity (for method, see V-2.1). Figures show mean bond strength ± standard error (n=3).

V-3.2 Bond strength of alternative Transglutaminases at a cartilage-cartilage interface

V-3.2.1 Microbial transglutaminase

V-3.2.1.1 Activa-EB

Figure V-9 shows the bond strengths produced by various Activa-EB preparations, and buffer control solution, at cartilage-cartilage interfaces after incubation under standard conditions.

Activa-EB is composed of 0-5% Ajinomoto microbial TG (from *Streptoverticillium*), 39.5% maltodextrin and 60% caesinate. An Activa-EB solution prepared using the manufacturer's recommended re-hydration ratio (containing 2.5mg/ml mTG assuming a 1% mTG content) showed a bond strength of 0.036 ± 0.005 MPa under standard conditions (Fig V-9). The highest bond strength achieved with Activa-EB was found using Activa-EB powder in the presence of Tris-buffered saline (0.086 ± 0.013 MPa), with Activa-EB powder alone producing a bond strength of 0.061 ± 0.005 MPa. Both of these results were significantly greater ($p<0.05$) than the bond strength found for Activa-EB solution.

The bond strength of a buffer control solution at equivalent interfaces following similar incubations for 20 minutes was 0.025 ± 0.004 MPa. Activa EB produced significantly greater bond strengths ($p<0.05$) than this when used as both a powder and as a powder with Tris buffered saline.

It was not possible to assay the activity of the Activa EB preparation by [^{14}C]-putrescine incorporation into N,N' dimethyl casein as the preparation contained a high level of caseinate itself. However, for comparison with the commercial tTG used in the previous bond strength studies, the activity of Activa EB is quoted as 0.05 U/mg (product literature) as measured by the hydroxamate method (Folk & Cole, 1966). The activity of the guinea-pig liver tTG (SIGMA) is quoted as 1.5-3 U/mg by the same method (product literature).

Figure V-9 Bond strength achieved at a cartilage-cartilage interface with use of various 'Activa-EB' microbial transglutaminase preparations and buffer control, under standard incubation conditions

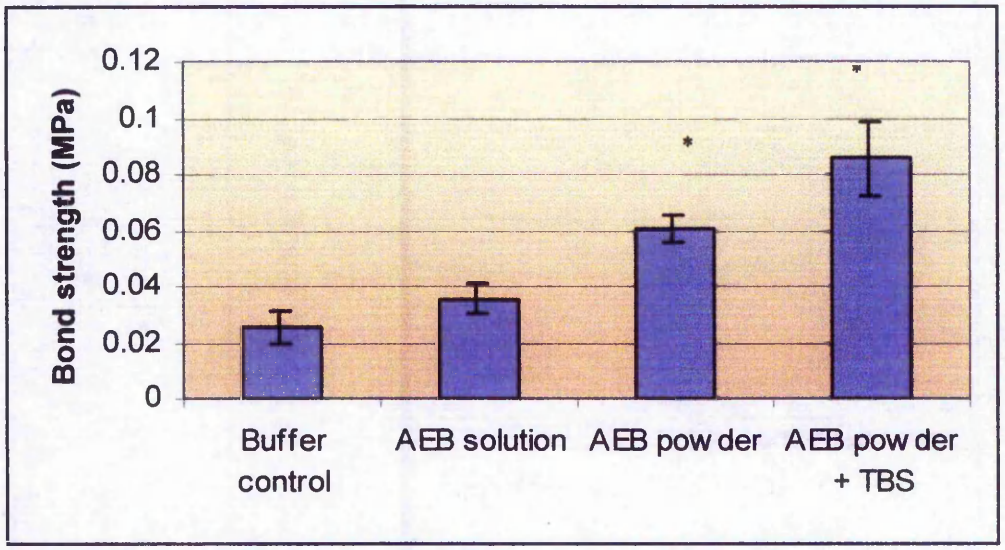

Figure V-9.

Bond strength of Activa-EB preparations and buffer control after incubation for 20min at 37°C, 20% humidity (for method, see V-2.2.1.1). Figures show mean bond strength ± standard error (n=4). Statistically significant data (vs. control) are marked as * ($p<0.05$).

Activa-EB preparations used at the cartilage-cartilage interface were:

1. 10µl Activa-EB solution (re-constituted according to manufacturers recommended re-hydration ration, 6-10g AEB + 20-30g H_20)
2. Activa-EB powder applied directly, sufficient to cover one surface of the cartilage-cartilage interface
3. Activa-EB powder applied directly, sufficient to cover one surface of the cartilage-cartilage interface and 10µl of Tris-buffered saline (pH 7.3) applied to the other.

V-3.2.1.2 KHM2

Activa KHM2 was an alternative Ajinomoto mTG preparation consisting of 90% maltodextrin and 10% mTG.

Solutions of KHM2 were analysed via SDS-PAGE (Figure V-10) and all show a major band between 29 and 34.6 Kda, which increases with increasing solution concentration.

A protein assay on a 10mg/ml (total weight/volume) solution of KHM2 gave a protein content of ~1mg/ml, which is as expected if the preparation does consist 10% mTG.

A pH of 6.5 was chosen for the bond strength studies of KHM2 (in terms of the buffer into which it was re-constituted and also combined with at the cartilage-cartilage interface), as this was in the middle of the pH range for Ajinomoto mTG activity (as stated in product literature). A KHM2 solution was proved to be active at pH 6.5, as assayed by [^{14}C]-putrescine incorporation into N-N'dimethyl casein. Though an activity in terms of incorporation (nmol/hr/mg) cannot be quoted as the sample is uninhibited by EDTA and so a positive control figure is unavailable. However, for comparison with the commercial tTG used in the previous bond strength studies, the activity of KHM2 is quoted as 1U/mg (product literature) as measured by the hydroxamate method (Folk & Cole, 1966). The activity of the guinea-pig liver tTG (SIGMA) is quoted as 1.5-3 U/mg by the same method (product literature). One unit is defined as catalysing the formation of 1 μ mole of hydroxamate per minute from N-α-CBZ-Gln-Gly and hydroxylamine.

The bond strength of KHM2 preparations was measured at a cartilage-cartilage interface under standard incubation conditions. Using solutions of 20, 40 and 80 mg/ml (total weight/volume, therefore 2, 4 and 8 mg/ml mTG assuming a 10% TG content) bond strengths of 0.037 ± 0.004, 0.034 ± 0.001 and 0.029 ± 0.002 MPa were achieved respectively (Fig V-11). There is no increase in bonding strength with increasing mTG content.

Figure V-10 SDS-PAGE analysis of the composition of 'Activa KHM2' microbial TG preparations

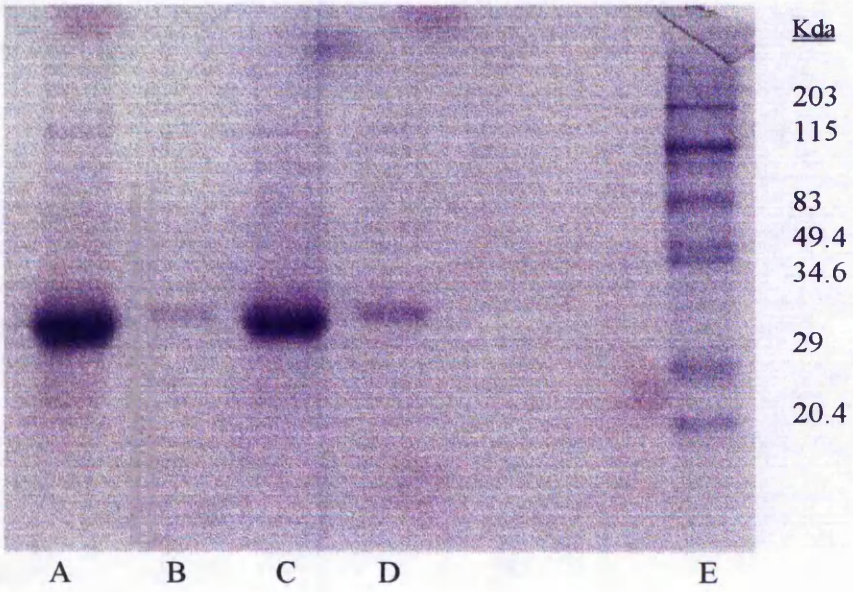

Figure V-10.

KHM2 solutions analysed via SDS-PAGE on a 10% acrylamide content resolving gel (for methods see II-5 and V-2.2.1.2). 10µl of KHM2 solution was run per lane using both 50 and 5mg/ml solutions (total w/v, therefore 5 and 0.5 mg/ml mTG content). Bands were visualised with Co-massie Blue stain.

Lane A - 50mg/ml (Total w/v) KHM2 solution (5mg/ml mTG) in 50mM Tris.Cl buffer (pH 7.3)

Lane B - 5mg/ml KHM2 solution (0.5mg/ml mTG) in 50mM Tris.Cl buffer (pH 7.3)

Lane C - 50mg/ml KHM2 solution (5mg/ml mTG) in 50mM Tris.Cl buffer (pH 6.5)

Lane D - 5mg/ml KHM2 solution (0.5mg/ml mTG) in 50mM Tris.Cl buffer (pH 6.5).

Lane E - Broad range molecular weight markers (BIO-RAD, UK)

Figure V-11 Bond strength of 'Activa KHM2' preparations at cartilage-cartilage interfaces, after incubation under standard conditions

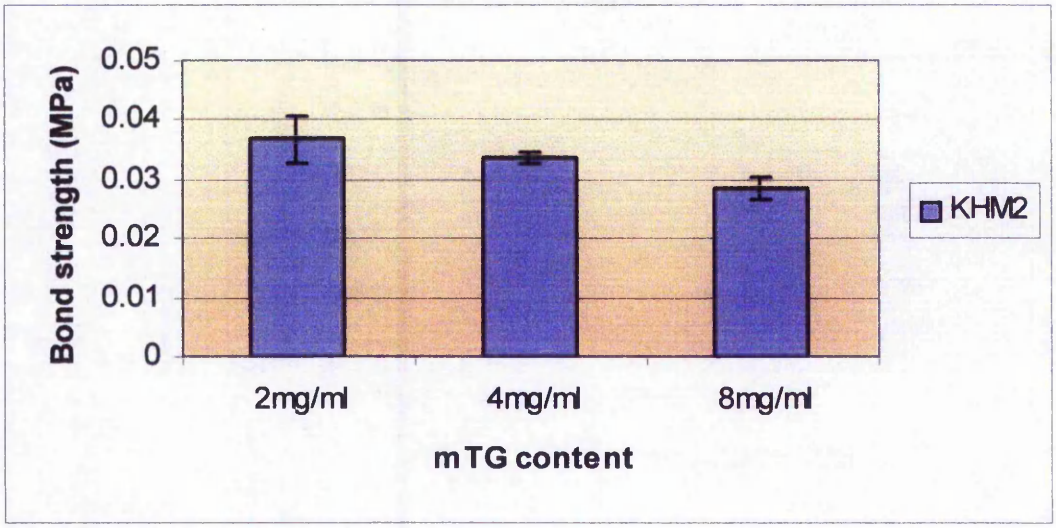

Figure V-11.

Bond strength of KHM2 preparations (2, 4 & 8mg/ml mTG content) after incubation for 20min at 37°C, 20% humidity (for method, see V-2.2.1.2). Figures show mean bond strength ± standard error (n=4).

Ammonium sulphate precipitation was performed on the KHM2 solution to purify the mTG by removing maltodextrin from the preparation (for method see V-2.2.1.2). The procedure resulted in a sample of 3mg/ml protein content (in 50mM Tris.Cl buffer pH 6.5) which was used under the assumption that this represented the mTG content (sample labelled 'A' here on). The activity of this sample was confirmed by assay ([^{14}C]-labelled putrescine incorporation into N'N-dimethyl casein). Though an activity in terms of incorporation (nmol/hr/mg) cannot be quoted as the sample is uninhibited by EDTA, therefore a positive control figure is unavailable.

The bond strength of Sample A was measured at a cartilage-cartilage interface and found to be 0.048 ± 0.004 MPa after incubation under standard conditions (5µl sample + 5µl 50mM Tris.Cl buffer (pH 6.5), Figure V-12, TAB was not used as mTG does not require calcium for its activation). This result is a higher bond strength than found for any of the previous KHM2 solutions which had theoretical mTG contents of 2, 4 and 8 mg/ml (Fig V-11).

Increasing the incubation time for sample A to 40 minutes gave a bond strength of 0.033 ± 0.003 MPa, which was less than that achieved after 20 minutes (Fig V-12). This is opposite to the trend observed for tTG bond strength in relation to incubation time (see III-3.1.3).

Some microbial transglutaminases are activated in the presence of NaCl, but application of Sample A in the presence of NaCl (0.3M) did not raise its bond strength at a cartilage-cartilage interface. A figure of 0.036 ± 0.005 MPa was achieved, compared to 0.048 ± 0.004 MPa in the absence of NaCl (Fig V-12).

Digesting the surfaces of the cartilage-cartilage interface to remove GAG's had a beneficial effect on the bond strength of Sample A, as it did for tTG, though the increase found was not significant. The preparation showed a bond strength of 0.036 ± 0.005 MPa at an untreated cartilage-cartilage interface, compared to 0.082 ± 0.014 MPa at an interface treated for 15 minutes with CAC (1U/ml) prior to mTG application (Fig V-12).

Figure V-12 Bond strength of a part purified microbial TG preparation (sample A) at untreated and GAG-digested cartilage-cartilage interfaces after incubation under standard conditions

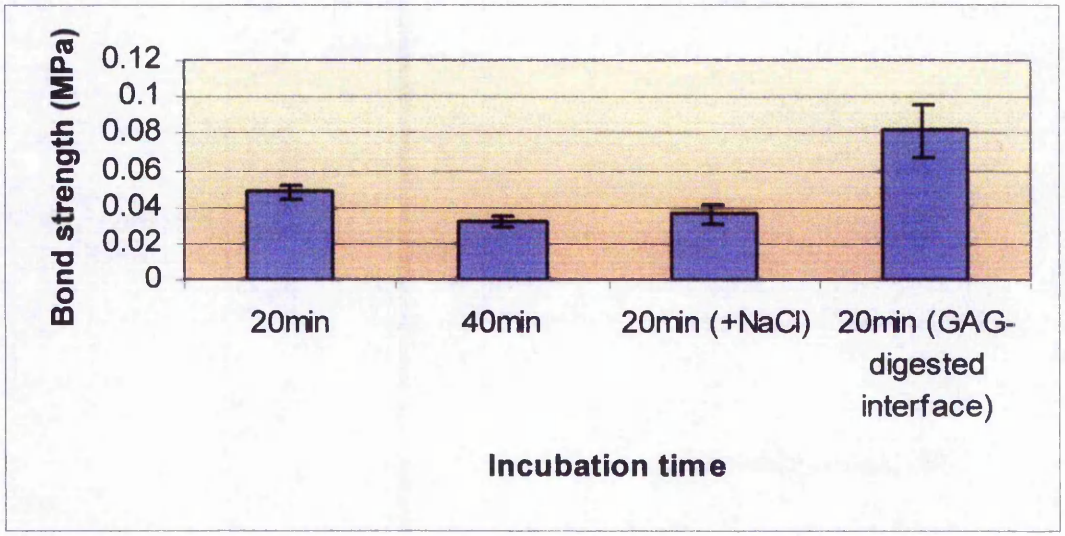

Figure V-12.

Bond strength of mTG solution (Sample A, 3mg/ml in 50mM Tris.Cl buffer (pH 6.5) produced following ammonium sulphate precipitation of 'KHM2' Ajinomoto microbial TG preparation, for method see V-2.2.1.2). Bond strength was determined after incubation for 20 or 40min at 37°C, 20% humidity. Interface was either untreated or digested with CAC (1U/ml) for 15 minutes prior to bonding (for method, see V-2.3.1.2). Figures show mean bond strength ± standard error (n=4).

V-3.2.2 Factor XIII

Factor XIII is also known as plasma transglutaminase and requires thrombin and calcium for its activation (see I-3.2.2). The Factor XIII used in this application was a commercial healthcare product 'Fibrogammin®' (Centeon, UK) which, once re-constituted according to manufacturers literature, consists of 62.5 U/ml of blood coagulation Factor XIII in H_20.

The bond strength shown by Fibrogammin® at a cartilage-cartilage interface, was 0.032 ± 0.002 MPa under standard incubation conditions (5µl Fibrogammin® + 5µl of TAB, Figure V-15). Increasing the incubation time did not lead to an increase in bond strength, as after incubation for 40 minutes Fibrogammin® gave a bond strength of 0.032 ± 0.003 MPa (Fig V-13). Neither of these two bond strengths were significantly different to those produced by the buffer control solution after similar incubations

The activity of Fibrogammin® was measured by [^{14}C]-putrescine incorporation into N'N-dimethyl casein and found to be approximately one hundredth of the activity of the tTG (1mg/ml) solutions assayed as a quality control measure (27 nmol/hr/mg specific activity compared to 2000-3000 nmol/hr/mg specific activity). However, the exact Factor XIII content of the preparation (in terms of protein) is not known (the assay was performed without the addition of thrombin). The protein content of Fibrogammin® was assayed as 8.75 mg/ml, but other proteins are included in the commercial preparation, including human albumin (for methods of determining Fibrogammin® protein content and activity see V-2.2.2).

The bond strength shown by Fibrogammin® was not altered when applied in the presence of thrombin/CaCl solution (in place of TAB solution). A bond strength of 0.028 ± 0.002 MPa was shown by Fibrogammin® plus 40mM CaCl containing 250U/ml Thrombin, after incubation under standard conditions (Fig V-13).

Figure V-13 Bond strength achieved at a cartilage-cartilage interface with use of Fibrogammin® and buffer control, applied in the presence of 'TAB' or CaCl+Thrombin

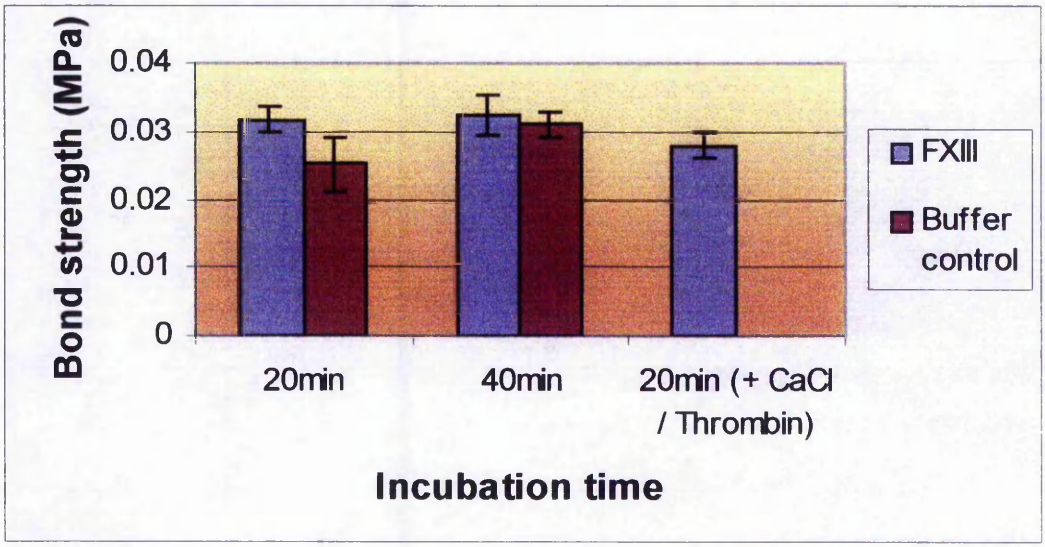

Figure V-13.

Bond strength of Fibrogammin® (FXIII) or buffer control after incubation for 20 or 40 minutes at 37°C, 20% humidity. 5µl of Fibrogammin® (62.5U/ml FXIII activity, 27 nmol/hr/mg specific activity) or buffer control was applied to the interface along with either 5µl of Tris Activating buffer 'TAB' (as standard) or 5µl of 40mM CaCl solution containing 250U/ml Thrombin (for method, see V-2.2.2). Figures show mean bond strength ± standard error (n=4).

V-3.3 Bond strength of tissue transglutaminase at GAG-digested cartilage-synthetic polymer interfaces

The bonding capacity of tTG was tested at interfaces between cartilage and synthetic polymers that have potential application as biomaterials. The cartilage surfaces were pre-treated with Chondroitinase AC (1U/ml) for 15 minutes prior to bonding, to remove GAG groups from the tissue surface.

Figure V-14 shows that tTG is capable of bonding synthetic polymer biomaterials to a GAG-digested cartilage surface. The biomaterials used were synthetic polymers, optimised to contain either ε-lysine residues (poly(lactic acid-co-lysine)) or ECM proteins (biocomposite of poly(ε-caprolactone) / fibronectin / collagen) for tTG cross-linking. This was to take advantage of the increased numbers of peptide bound γ-glutamyl residues that GAG-digestion had exposed on the cartilage surfaces, maximising available cross-linking sites at the interface and aiding tTG-catalysed bonding.

Tissue transglutaminase (4mg/ml) showed a bond strength of 0.052 ± 0.004 MPa at an untreated cartilage-cartilage interface and 0.066 ± 0.008 MPa at a GAG-digested cartilage-cartilage interface, after incubation under standard conditions (Fig V-14). Both of these results were significantly greater ($p<0.05$) than the bond strengths achieved with use of the buffer control solution.

At an interface between GAG-digested (CAC 1U/ml 15min) cartilage and poly-lactic acid-co-lysine, tTG (4mg/ml) produced a bond strength of 0.053 ± 0.008 MPa. Compared to 0.034 ± 0.004 MPa for the buffer control at an equivalent interface. This is a significant difference ($p<0.05$).

At an interface between GAG-digested (CAC 1U/ml 15min) cartilage and poly(ε-caprolactone) / fibronectin / collagen, tTG (4mg/ml) produced a bond strength of 0.040 ± 0.002 MPa. Compared to 0.029 ± 0.002 MPa for the buffer control at an equivalent interface. This is a significant difference ($p<0.05$)

Figure V-14 Bond strength of tissue transglutaminase and buffer control at various interfaces, after incubation under standard conditions

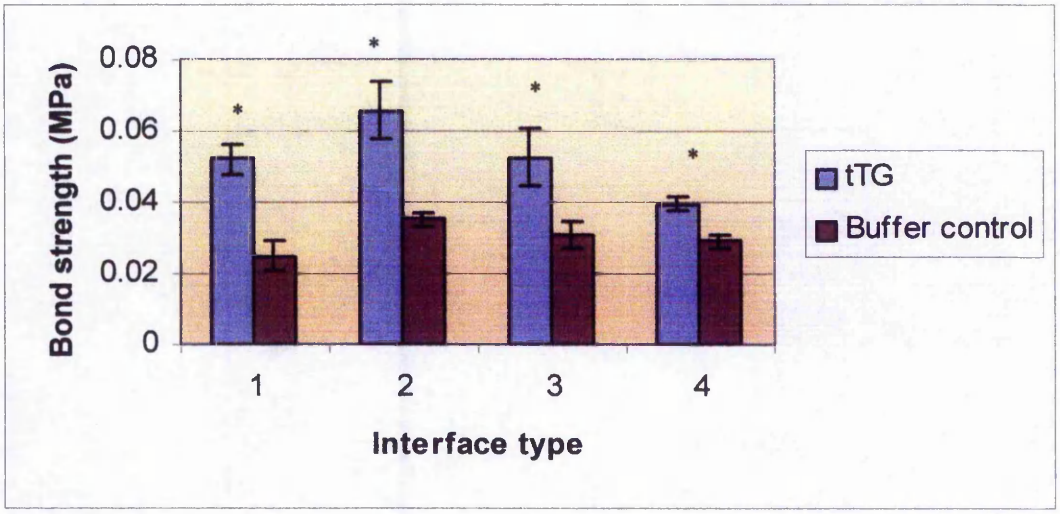

Figure V-14.

Bond strength of tTG (4mg/ml, 3444 nmol/hr/mg specific activity) and buffer control at:

1. Untreated cartilage - cartilage interface
2. GAG-digested cartilage - GAG-digested cartilage interface
3. GAG-digested cartilage – poly (lactic acid-co-lysine) interface
4. GAG-digested cartilage – poly(ε-caprolactone) / fibronectin / collagen interface

Incubations preceding determination of bond strength were for 20min at 37°C, 20% humidity. GAG-digestion occurred using CAC (1U/ml) for 15 minutes at 37°C prior to bonding (for methods, see V-2.3). Figures show mean bond strength ± standard error (n=4). Statistically significant data (tTG-treated samples compared to buffer control at each interface type) are marked as * ($p<0.05$).

V-4 Conclusions

Whilst investigating the effects of additional proteins on the bond strength of tTG preparations, it was found that a protein thickening agent could be added to the adhesive with no loss of bonding strength. Thus if the fluid nature of the tTG preparation becomes a problem for the surgical user, i.e. in retaining the solution at the site of application, a thickener can be added with no detrimental effect on bonding capabilities. In this case the thickener was pullulan, a polysaccharide used for similar effect in dermatological lotions.

Attempts to raise the bond strength of tTG preparations by the inclusion of tTG-substrate proteins or polyamines in the adhesive preparation were largely unsuccessful.

The inclusion of polyamines, bovine albumin, casein, fibronectin and poly-l-lysine in a tTG preparation all failed to improve its bonding capabilities at untreated cartilage-cartilage interfaces. Proteins were also added to tTG preparations applied to GAG-digested interfaces to see if they could raise bonding strength. More specifically, lysine-rich proteins were included to provide increased ε-lysine residues for tTG to cross-link with the increased numbers of exposed γ-glutamyl residues found on GAG-digested cartilage surfaces. The intention was to again raise the amount of cross-linking between the digested interface surfaces and promote bonding. However, the inclusion of casein, putrescine and poly-l-lysine in a tTG preparation also failed to improve its bonding capabilities at GAG-digested cartilage-cartilage interfaces.

Additional proteins that did have a beneficial effect included gelatin. This is both a tTG-substrate and a gel-forming protein in solution at room temperature. The inclusion of gelatin significantly raised the bond strength of tTG (4mg/ml) at a cartilage-cartilage interface from 0.054±0.004 MPa to 0.075±0.006 MPa. A similar significant increase was not observed in the buffer control. This suggests that the beneficial effect of gelatin in conjunction with tTG was due to it being a tTG substrate, not its ability to gel in solution at room temperature. Since testing was performed at room temperature, any positive effect gelling may have had on the bond strength and bonding the interface together should have been observed in the control solutions also and this was not the case.

Gelatin is already being investigated as the basis for a biological adhesive (see I-2.4) and gelatin cross-linked by TG has also been proposed as a system for enclosing bioactive materials without a detrimental effect on their activity (Fuchsbauer *et al*, 1996). Thus a tTG/gelatin based adhesive may not only help bond interfaces during cartilage repair, but could also be used to deliver bioactive molecules (e.g. growth factors) to the defect site to promote repair processes.

Collagen II also improved the bond strength of tTG at cartilage-cartilage interfaces, though this increase was not significant. The acid-soluble collagen was re-constituted in 0.1M acetic acid and added to the tTG preparation before application to the interface. Despite the possible alteration of the pH of the enzyme solution, the bond strength of tTG (4mg/ml) increased from 0.036 ± 0.001 MPa to 0.063 ± 0.012 MPa in the presence of collagen II (2.5mg/ml).

The theory of using a protein additive to raise bonding strength of a tTG preparation still seems a sound one, despite the limited success reported so far.

In food adhesive preparations, protein powder (preferably a milk protein such as casein) is used to exhibit a strong adhesion strength upon combination with the active ingredient of either a calcium-dependent or calcium-independent TG (Yasuyuki, 1996). Also polypeptides cross-linkable by transglutaminase, comprising a glutamine and lysine residue separated by a spacer peptide, have been proposed as ingredients in tissue sealant and wound healing formulations (Labroo *et al*, 1995). In which their cross-linking may add stability to a biomaterial and enable it to adhere to tissue surfaces. Therefore the addition of such a specifically designed and synthesised polypeptide may succeed in raising the bond strength of tTG preparations at cartilage-cartilage interfaces.

The bond strengths of alternative transglutaminases were also investigated at cartilage-cartilage interfaces.

A commercial preparation, 'Activa-EB', containing 0-5% Ajinomoto microbial TG (from *Streptoverticillium*) along with caseinate and maltodextrin produced a bond strength of 0.036 ± 0.005 MPa at a cartilage-cartilage interface when used as a solution at the recommended re-hydration ratio (under standard incubation conditions). The preparation is intended as a meat-binding product and can be used in

powder form alone if the tissue to be bound has a high water content. As cartilage does have a high water content, the bond strength of Activa-EB powder was also tested and found to be 0.061 ± 0.005 MPa under standard conditions. This is significantly greater ($p<0.05$) than the bond strength of Activa-EB solution, as was the bond strength of Activa-EB powder in the presence of TBS. The presence of TBS seemed to increase the bonding strength of Activa-EB powder (though this was not statistically significant) suggesting that the mTG may be activated by the presence of NaCl, as was thought. Or the fluid may simply aid the Activa-EB powder in covering the interface surfaces.

Whilst the actual mTG content of these preparations remains unknown, comparing these bond strengths to those found for tTG remains difficult. But a bond strength of 0.061 ± 0.005 MPa (as was found for the Activa-EB powder) is equivalent to that produced by tTG preparations of at least 4mg/ml, under equivalent conditions. Activa-EB produced bond strengths significantly greater ($p<0.05$) than those of a buffer control under similar conditions when used as both a powder and as a powder in the presence of Tris buffered saline.

In an effort to be more certain of the mTG content of the preparation being used, a second product 'KHM2' was used which contains 10% Ajinomoto mTG (from *Streptoverticillium*) and 90% maltodextrin. Protein assays on samples of KHM2 solution showed a protein content that equates with the proposed contents of 10% mTG and 90% maltodextrin stated in the product literature. SDS-PAGE analysis of KHM2 solutions showed a major band, thought to represent mTG, of between 29,000 and 34,600 Kda in size, which is less than the stated Mr of 38,000 (Ando *et al*, 1989).

The bond strength of KHM2 solution at a cartilage-cartilage interface did not show an increase with increasing mTG content. Assuming the preparation to be 10% mTG, 2, 4 and 8 mg/ml mTG solutions gave bond strengths of 0.037 ± 0.004, 0.034 ± 0.001 and 0.029 ± 0.002 MPa respectively after incubation under standard conditions.

Ammonium sulphate precipitation, to remove maltodextrin from the KHM2 solution, produced a sample of 3mg/ml-protein content, which was assumed to be the mTG content. The bond strength of this sample was found to be higher than those found for the previous KHM2 preparations, but bond strength did not increase with increasing incubation time. The maltodextrin may have been present in the preparation to stop

the mTG from cross-linking itself before application (as it requires no calcium for activation). Auto-cross-linking may then have occurred to the sample following the removal of maltodextrin, which caused the preparation to lose activity over time. As was found for tTG, digestion of surface GAG's from the cartilage interface enhanced the bond strength of the precipitated mTG sample, though this was not found to be statistically significant. Residual ammonium in the mTG preparation (remaining from the ammonium sulphate precipitation) may also have inhibited the enzyme activity since it is part of the products of the cross-linking reaction.

The bond strength of a commercial Factor XIII preparation (Fibrogammin®) was also investigated. This health-care preparation is conventionally administered to patients with blood-clotting deficiencies and whilst the units of activity of Factor XIII present in the sample were known (in terms of blood-clotting activity), again the actual concentration of TG was not, making comparison of bond strengths to those found for tTG difficult. Other proteins, such as human-albumin, are included in the formulation and so comparing the adhesive strength of tTG to that of Factor XIII would need to be done using a solution of known FXIII content, so that equal amounts, or levels of activity, of the two enzymes may be employed to bond the cartilage-cartilage interfaces. Fibrogammin® bond strength was not found to be significantly greater than those of the buffer control, over both 20 and 40-minute incubations. Another uncertain factor was the activation state of the Factor XIII, i.e. was it Factor XIII or Factor XIIIa after activation by the action of thrombin. To this end, the bond strength of Fibrogammin® was measured in the presence of calcium chloride including thrombin, but this had no beneficial effect on raising bonding strength compared to Fibrogammin® used with Tris Activating Buffer.

Results have also demonstrated the tTG-catalysed bonding of an interface between cartilage and biomaterial, both of which were optimised for the purpose of achieving maximum bond strength. The cartilage was subjected to GAG-digestion to expose peptide-bound γ-glutamyl residues and the biomaterial was synthesised to include ε-lysine groups or ECM proteins that were available for TG-catalysed cross-linking to the exposed γ-glutamyl residues.

The bond strength found for tTG (4mg/ml) at the interface between GAG-digested cartilage and biomaterial was significantly greater than that found for buffer control solution at equivalent interfaces. The biomaterials investigated were poly-lactic acid, synthesised to include multiple ε-lysine groups, and poly(ε-caprolactone) synthesised to include the ECM protein's collagen and fibronectin. Poly-lactic acid is a synthetic matrix that is generally more frequently employed in orthopaedic applications than poly(ε-caprolactone), due to its higher mechanical strength. It has already had application in cartilage repair, as a matrix for the reconstruction of articular defects in the rabbit (Von Schroeder *et al*, 1991), and has also found application as resorbable sutures (Benicewicz & Hopper, 1991) and bone fixation screws and plates (Rizzi *et al*, 2001). Poly(ε-caprolactone) has been used for orthopaedic applications, such as the repair of bone defects (Coombes & Meikle, 1994). It is a semi-crystaline polymer with high solubility and degrades at a slower pace than poly-lactic acid and can therefore also be used in drug delivery devices that remain active over long periods of time. It is currently regarded as non-toxic and tissue compatible and is in clinical use as a degradable staple in Europe.

These results, along with those presented in chapter III, show that tTG is capable of bonding the following interfaces with significantly greater bond strengths than those shown by a buffer control solution:

Untreated cartilage – to – Untreated cartilage

GAG-digested cartilage – to – GAG-digested cartilage

GAG-digested cartilage – to – Poly(lactic acid-co-lysine)

GAG-digested cartilage – to – Poly(ε-caprolactone) / fibronectin / collagen I.

Biomaterials are becoming an increasingly important aspect of cartilage repair as they are implanted into defect sites to act as scaffolds for the remodelling of the defect site by implanted or host cells. Retention of scaffolds in defect sites has proved problematic, but these results now demonstrate that tTG can be used to bind biomaterials to cartilage surfaces and help their retention at the site of application. The biomaterials in question can then be optimised both for the ability to act as a cell

scaffold, and for their retention in defect sites by tTG-catalysed cross-linking through the inclusion of multiple tTG substrate residues in the material structure.

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VI-1 Introduction

Following investigation of TG-catalysed bonding at cartilage-cartilage interfaces, the levels of cell viability at wound edges within cartilage tissue were also studied.

The analysis of experimental defects in articular cartilage has revealed a wound response characterised by a zone of cell death at the wound edge, which can extend to four to five cell widths on either side of the injury (Mankin, 1962). This band is seen as early as 1-day post-wounding and has largely been attributed to necrosis (Mankin, 1962). However it has also been suggested that apoptosis may play a role (Tew *et al*, 2000). These studies also reported the occurrence of a proliferative response from surviving chondrocytes in the cell death area, and a zone of cellular proliferation alongside the zone of cell death (Tew *et al*, 2000; Mankin, 1962). This increase in mitotic activity has been associated with enhanced rates of cellular synthesis of matrix components (Mankin, 1962). But these responses are not sufficient to produce sufficient numbers of new cells or matrix to repair even a minimum defect (Mankin, 1962).

Whatever the precise nature of the mechanism of cell death, this loss of chondrocyte viability at wound edges may have serious detrimental effects on cartilage repair.

The lack of integration between host and repair tissues has already been discussed as a major problem in the field of cartilage repair (see I-2.5). Uninterrupted continuity between repair and native tissue must be established from the onset and maintained (Hunziker, 1999b) and this adhesion and integration is often a clinical limitation in current cartilage repair technologies. It is likely that the loss of viable cells at wound edges is a contributing factor in this lack of integration. In a study of full-thickness defects in the articular cartilage of New Zealand white rabbits, areas of necrotic, non-viable cartilage adjacent to the defect drill-holes were observed, that were 3-10 cell columns wide (Shapiro *et al*, 1993). The resulting empty lacunae remained empty over a 48-week period and thus a small, circumferential band of cartilage composing the wall of the defect remained dead and its extra-cellular matrix wasn't replaced or

repaired. A possible reason for the subsequent failure of the repair tissue formed in the defect was suggested to be the quality of its physicochemical binding to the adjacent native tissue. This may have been deleteriously influenced by the band of dead tissue around the wall of the defect.

Cartilage repair technologies may therefore be improved by:

a) the development of a method for integration between repair and native tissue (a problem addressed in this thesis with the investigation of a biological cross-linking agent for cartilage interfaces, based on transglutaminse) and also

b) The possible reduction and attenuation of the cell-death that occurs at cartilage wound edges. Since even if integration is achieved, it may be to a largely dead area of native tissue. A fact that may compromise the resulting repair tissue formed by any number of cartilage repair strategies, such as soft tissue grafts, implanted matrices, implanted cell populations and chondral/osteochondral grafts.

The initial aim of the work in this chapter was to see how the proposed methods for aiding bonding and integration at cartilage-cartilage interfaces (tTG-catalysed cross-linking or GAG-digestion) might influence the degree of cell death seen at cartilage wound edges, whether this be a positive or negative effect. The secondary aim of the work in this chapter was to undertake preliminary investigations into ways of reducing/inhibiting the cell death that occurs at cartilage wound edges.

Using an *in vitro* articular cartilage explant culture system, the degree of cell death occurring at experimental wound edges was measured over time in response to certain variables and treatments of the wound edge.

A two-colour fluorescence cell viability assay (Molecular Probes, USA) was used that simultaneously determines live and dead cells, with two probes that measure recognised parameters of cell viability. Live cells are distinguished by the presence of intracellular esterase activity, which converts the non-fluorescent cell-permeant Calcein AM to fluorescent calcein. Calcein is then retained by the live cells, which produce a uniform green fluorescence. The second probe (Ethidium homodimer-1) is based on plasma membrane integrity. It enters cells with damaged membranes and undergoes an enhancement of fluorescence upon binding to nucleic acids, producing a red fluorescence in dead cells.

The articular cartilage explant culture system was used to determine the degree of cell death occurring at experimental wound edges over time, in response to tTG-catalysed bonding, GAG-digestion, the presence of serum, contact with surrounding tissue, oxygen levels in culture and the presence of caspase inhibitor.

Contact with surrounding tissue was investigated to see if maintaining close contact and some degree of integration between wound edges following injury could help reduce the loss of chondrocyte viability. If chondrocytes are dying due to the lack of a possible 'survival signal' from surrounding cells or matrix, then keeping the cells at the wound edge in close approximation to the surrounding tissue might help preserve this signal and keep more chondrocytes viable.

The effects of oxygen levels and the presence of serum were investigated to see how cartilage might be stored *in vitro* to minimise loss of cell viability. Since long-term survival of cartilage grafts depends on the continued viability of its cells (Newman, 1998) it is prudent to ensure that there is the maximum possible number of viable chondrocytes within the graft tissue to begin with. Cartilage is an avascular tissue with low oxygen tension *in vivo*, and so reduced oxygen conditions may be effective in preserving chondrocyte phenotype and viability *in vitro*.

These studies might also elucidate the mechanism of cell death occurring at cartilage wound edges, which was the rationale behind the investigation of the effects of caspase inhibitor also. Caspases are a family of enzymes that play a critical role in many of the morphological and biochemical changes associated with apoptosis (Cohen, 1997; Earnshaw *et al*, 1999). If apoptosis is a contributory mechanism in the loss of chondrocyte viability at cartilage wound edges then the application of a caspase inhibitor may reduce this. Which may then help promote successful integration between native and repair tissue by increasing the number of viable chondrocytes at the wound edge. The inhibitor might conceivably be applied using a tTG-based adhesive as the delivery vehicle. Whose cross-linking action could promote tissue integration and also help retain the inhibitor at the site of application.

VI-2 Methods

VI-2.1 Articular cartilage explant culture

Porcine limbs were obtained from local butchers (Alan Knight, Nottingham, UK; Robin Tuxford, Nottingham, UK) and used in experiments on the day of collection.

Full-depth cartilage explants (~ 5 x 10 mm) were dissected from the metacarpal/metatarsal joints, with care taken not to excise calcified tissue.

Explants were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 2 mM L-glutamine and 50 U/ml penicillin / 50 μg/ml streptomycin. Unless stated otherwise this was the standard tissue culture medium used for all experimentation. Media was pre-acclimatised to 37°C, 5% CO_2 before the explants were added, and the explants were then cultured under these conditions unless stated otherwise. Explants were cultured in 12-well plates (1 per well), with 3mls media per well and media was changed every other day.

VI-2.2 Creation of experimental wound edges within cultured articular cartilage explants

Explants were cultured for 2 hours at 37°C, 5% CO_2 before being wounded (whilst remaining in media) using a sterile biopsy punch (internal diameter 2mm, Stiefel, UK). Wounding occurred from the articular surface downward and two wounds were made per explant, creating two 'punches' of tissue. These tissue punches were either left in contact with the rest of the explant or removed to float freely in the culture media.

Within each explant, one tissue punch was used as a control, which was defined as being created and removed from the explant to be cultured in standard media, under standard conditions. The degree of cell death measured at the wound edge of the second tissue punch from the explant (treated in various ways to assess the effects on cell viability) was then compared to that seen at the wound edge of the control punch.

Tissue punches and explants were cultured and harvested at either 1, 5 or 7 days post-wounding to measure the degree of cell death at their wound edges. In general, four explants were harvested at each time point.

VI-2.3 Assessing cell viability at experimental wound edges within cultured articular cartilage explants

The viability of cells, at the experimental wound edge surrounding the cartilage punches, was determined using confocal laser scanning microscopy (CLSM).

At each time point, tissue punches were stained using a 'LIVE/DEAD®' Viability/Cytotoxicity Kit (Molecular Probes, Oregon, USA).

A combined stain solution was prepared from Calcein AM (4 mM in anyhydrous DMSO) and Ethidium homodimer-1 (2 mM in DMSO/H_2O (1:4 v/v)) stocks, diluted 1:100 in PBS. Each tissue punch was incubated in 25µl of the combined stain solution (in a microfuge tube) for 1 hr at 37°C, protected from light. Tissue punches were then rinsed in PBS and mounted within the individual chambers of a rubber gasket placed on a microscope slide. PBS was added to each chamber within the gasket and the punches were viewed using a LECIA TCS-NT confocal microscope with an argon krypton laser adjusted for FITC and CY5 excitations. Tissue punches were viewed with the articular surface uppermost, using a x10 objective. Images were taken at a depth of 100µm down into the tissue from the articular surface. A sample image of an experimental articular cartilage wound edge stained for cell viability using this method is shown in figure VI-1B.

The degree of cell death at the experimental wound edge was determined using image analysis software.

The distance that the band of cell death had progressed from the wound edge (toward the centre of the tissue punch) was measured at 8 points around the circumference of each punch (see Fig VI-1A). An average of this data was recorded as the width of the cell death band at each wound edge (as displayed on histograms ± standard error of the mean).

This result was compared to the width of the band of cell death seen in the control tissue punch, for each explant.

The data for each explant was compared by two-way analysis using the students' t-test (assuming unequal variance). Significant differences ($p<0.05$) versus controls are labelled as *.

To compare the effects of the various conditions investigated (see VI-2.4) on cell viability at experimental articular cartilage wound edges, the width of the band of cell death at each treated wound edge was expressed as a % of that measured at the wound edge of its respective control tissue punch. This data was averaged for condition, at each time-point and displayed on the figure as the average of 3 or 4 explants ± standard error of the mean. The data for each condition (treated wound edges vs. controls) was compared by two-way analysis using the students' t-test (assuming unequal variance). Significant differences ($p<0.05$) versus controls are labelled as *.

VI-2.4 Variables investigated to study their effect on chondrocyte viability at an experimental wound edge

VI-2.4.1 Presence of serum in the culture media

Within each explant, one punch was removed to be cultured in standard media and the other was removed and cultured in serum-free media.

VI-2.4.2 Contact with the surrounding explant tissue

Within each explant, one punch was removed to be cultured under standard conditions and the other was left within the explant after wounding (and the tissue plus punch subsequently cultured under standard conditions).

VI-2.4.3 Digestion of glycosaminoglycan groups (before and after culture)

Digestion post-wounding and before culture - Within each explant, one punch was incubated in Chondroitinase AC (1U/ml) for 15 minutes at 37°C prior to culturing (control was incubated in 50mM Tris.Cl buffer (pH 7.3) in place of CAC). Both punches were then rinsed in PBS before being cultured under standard conditions.

Digestion post-wounding and post-culture - Within each explant, both punches were removed and cultured under standard conditions. Prior to viewing, one punch was incubated in Chondroitinase AC (1U/ml) for 15 minutes at 37°C (control was incubated in 50mM Tris.Cl buffer (pH 7.3) in place of CAC). Both punches were then rinsed in PBS before viewing.

VI-2.4.4 Oxygen tension

Explants were cultured for 2 hours before wounding (as stated, VI-2.2) but at 5% O_2 levels rather than 20% O_2. Within each explant, one punch was then removed and cultured under standard conditions (37°C, 5% CO_2, 20% O_2) whilst the other was removed and cultured in standard media at 37°C, 5% CO_2 and 5% O_2.

VI-2.4.5 Presence of caspase inhibitor

Prior to wounding, each explant was cultured for 2hr under standard conditions in standard media containing 20μM Z-VAD-FMK (Promega, Madison, USA). Within each explant (post-wounding), one punch was then removed and cultured under standard conditions. The other was removed and cultured in standard media containing 20μM Z-VAD-FMK at 37°C, 5% CO_2.

Z-VAD-FMK is supplied as a 20mM solution in DMSO.

VI-2.4.6 Bonding of punches back into explant with tTG

Within each explant, one punch was removed and incubated in tTG solution (4mg/ml in 50mM Tris.Cl (pH 7.3) containing 50mM EDTA) for 20min at 37°C, before being placed back into the explant. Control punch was incubated in 50mM Tris.Cl buffer (pH 7.3) in place of tTG, before also being returned to the explant Explants were then cultured under standard conditions.

VI-2.5 Incorporation of Texas Red cadaverine into articular cartilage sections – a measure of Transglutaminase levels on the section surface

Articular cartilage explant was harvested as stated (see VI-2.1) and flash-frozen in liquid nitrogen into OCT (BDH Laboratory Supplies, Poole, UK) on a steel cryostat chuck.

Sections (10μm thick) were then cut using a cryotome operating at -18°C. Sections were adhered to Superfrost Plus Gold slides (BDH Laboratory Supplies, Poole, UK) and stored at -70°C until use.

Samples slides were thawed at room temperature and each tissue section circled using a hydrophobic pen (Vector Laboratories Inc, USA).

The following protocol used was amended from a protocol of Matthew Jamie (The Nottingham Trent University), for the measurement of *in situ* tissue transglutaminase activity.

50μl of reconstitution buffer was applied to each section for 2 minutes at room temperature. Reconstitution buffer stock solution comprised:

899μl 50mM Tris.Cl buffer (pH 7.3) / 3% BSA

50μl 20mM EDTA (in 50mM Tris.Cl (pH 7.3))

50μl Goat Serum

1μl Triton x-100

Each section was then washed for 3 minutes at room temperature in 50µl of wash solution. Wash solution stock comprised:

750µl 50mM Tris.Cl buffer (pH 7.3)

250µl 20mM EDTA (in 50mM Tris.Cl (pH 7.3))

Each section was then washed for 2 minutes at room temperature in 50mM Tris.Cl (pH 7.3).

Each section was then incubated with either 20µl of tTG solution (1mg/ml or 4mg/ml plus 50mM EDTA) or 50mM Tris.Cl (pH 7.3), for 20 minutes at 37°C in a slide humidity chamber.

These solutions were removed before the addition of either:

- 497.5µl Standard media (see VI-2.1) + 2.5µl Texas Red Cadaverine (1mM in 10mM Tris.Cl (pH 7.3)) (Molecular Probes, Oregon, USA)
- 447.5µl Standard media, 2.5µl Texas Red Cadaverine, 50µl 200mM EDTA (in 50mM Tris.Cl (pH 7.3)) (EDTA positive control, to sequester calcium from media and inhibit tTG activity)
- 500µl Standard media (no TC)

Samples were then incubated at 37°C for 30 minutes in a slide humidity chamber.

All sections were then washed in PBS (x2) and methanol (-20°C) before being mounted and viewed using a LEICA TCS-NT confocal microscope, with a x40 objective and an argon krypton laser adjusted for CY5 excitation.

Figure VI-1 A) Schematic diagram of the measurement of the degree of cell death occurring at experimental wound edges within articular cartilage explant tissue punches, B) Sample image of an articular cartilage tissue punch wound edge, stained for cell viability and viewed using CLSM

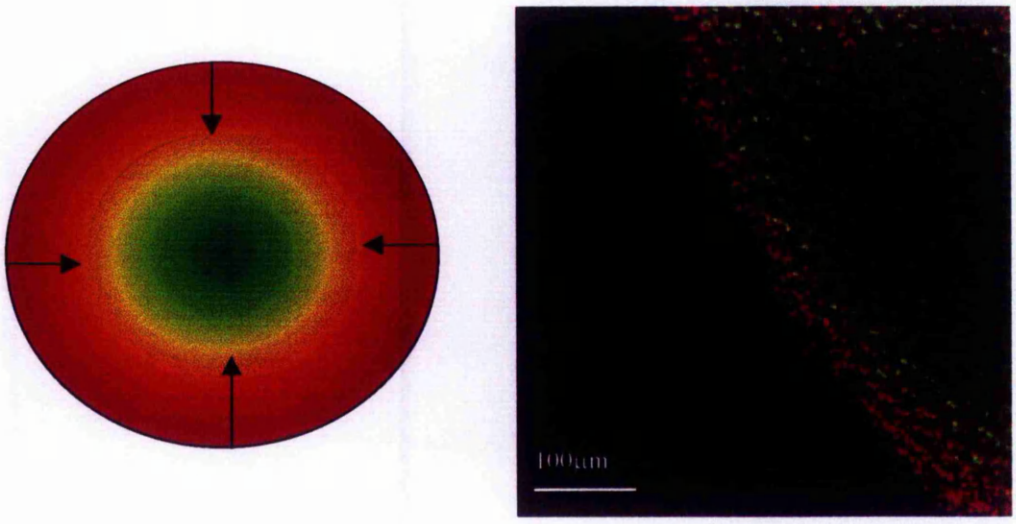

Figure VI-1A.

Articular cartilage tissue punches are viewed from above with the articular surface uppermost. The distance that the band of dead cells (fluorescing red) has progressed from the wound edge toward the centre of the sample is measured at eight points around the circumference of the tissue punch. An average of this figure is then taken as the width of the cell death band at that wound edge. Viable cells fluoresce as green.

Figure VI-1B.

Cell viability at an experimental articular cartilage wound edge. An articular cartilage explant was wounded *in vitro* using a biopsy punch and the tissue punch cultured for 5 days at 37°C, 5% CO_2 before being stained using a dual cell-viability stain according to the methods described (see VI-2.1, 2.2, 2.3). The tissue punch was then viewed using CLSM (x10 magnification) with the articular surface uppermost and images of the wound edge taken at a depth of 100µm from the articular surface. Non-viable cells show as red and viable cells show as green.

VI-3 Results

VI-3.1 Cell viability at experimental wound edges within cultured articular cartilage explants

VI-3.1.1 Effect of serum

The loss of cell viability at an articular cartilage wound edge was reduced when the tissue was cultured in the presence of foetal calf serum (Fig VI-2). This effect was more evident 7 days after wounding than 1-day.

At 1 day post-wounding, 2 out of 4 samples showed significantly ($p<0.05$) wider zones of cell death at the wound edges of tissue punches cultured in serum-free media, compared to controls cultured in media containing 10% FCS. Although one sample did show an opposite trend and more cell death at the wound edge cultured in media containing serum.

At 7 days post-wounding, 3 out of 4 samples showed significantly ($p<0.05$) wider bands of cell death at the wound edges of tissue punches cultured in serum-free media, compared to controls cultures in media containing 10% FCS.

For both serum-containing and serum-free conditions, the widths of the bands of cell death seen at the experimental wound edges tended to increase over time (7 days post-wounding compared to 1-day post-wounding).

Figure VI-2 Width of the band of cell death seen at experimental wound edges in articular cartilage cultured over 7 days post-wounding – effect of the presence of serum

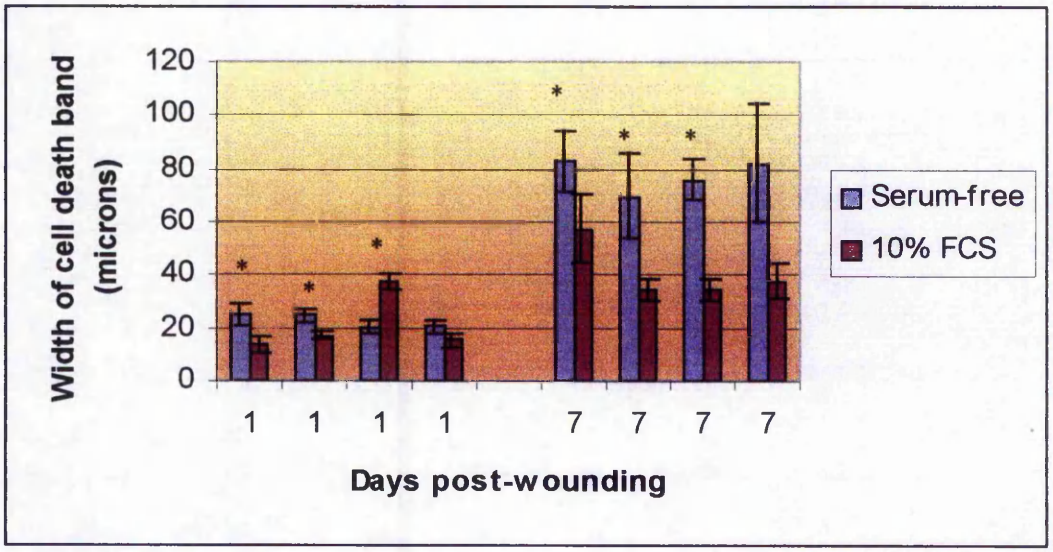

Figure VI-2.

Articular cartilage explants were wounded twice and the tissue punches removed and cultured in either standard media or standard media without serum, at 37°C, 5% CO_2 for 1 and 7 days post-wounding. Four explants were harvested at each time point and the widths of the bands of cell death at each wound edge were measured and compared according to the method described in VI-2.3 (figures show average of 8 measurements ± standard error of the mean). Statistically significant data (with serum compared to without) are marked as * ($p<0.05$).

VI-3.1.2 Effect of contact between the wound edge and surrounding explant tissue

The loss of cell viability at an articular cartilage wound edge was reduced when the wound edge was cultured in contact with the surrounding explant tissue (Fig VI-3).

For all samples at 1, 5 and 7 days post-wounding, the band of cell death at the wound edge was significantly ($p<0.05$) wider for control tissue punches removed to media compared to tissue punches that were not removed and were cultured in contact with the surrounding explant tissue.

For both punches removed to media and those left in contact with the surrounding tissue, the widths of the bands of cell death seen at the experimental wound edges tended to increase over time (from 1-day post-wounding through to 7 days post wounding).

Figure VI-3 Width of the band of cell death seen at experimental wound edges in articular cartilage explants cultured over 7 days post-wounding – effect of contact with surrounding tissue

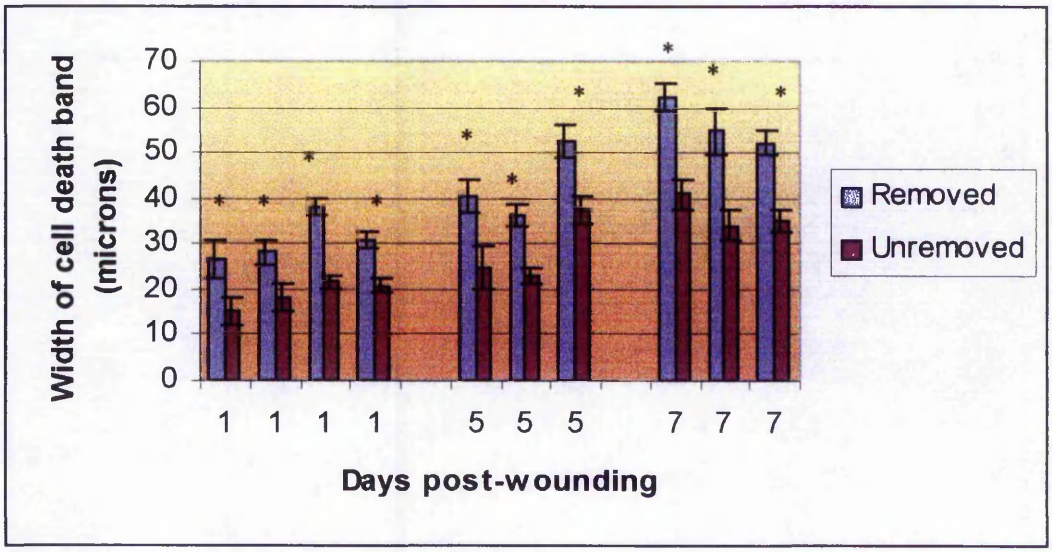

Figure VI-3.

Articular cartilage explants were wounded twice and one tissue punch was left in contact with the surrounding explant tissue whilst the other was removed. Both were cultured in standard media at 37°C, 5% CO_2 for 1, 5 and 7 days post-wounding. Four explants were harvested at day 1 and three at each of day 5 and day 7. The widths of the bands of cell death at each wound edge were then measured and compared according to the method described in VI-2.3 (figures show average of 8 measurements ± standard error of the mean). Statistically significant data (removed compared to unremoved) are marked as * ($p<0.05$).

VI-3.1.3 Effect of digestion of glycosaminoglycan groups

The loss of cell viability at an articular cartilage wound edge was increased when the wound edge was enzymatically digested to remove glycosaminoglycan groups.

GAG-digestion performed on the wound edge immediately after wounding and prior to culture for 1 or 7 days (Figure VI-4)

For tissue samples subjected to GAG-digestion post-wounding and prior to culture, all samples at 1-day post-wounding showed a significantly ($p<0.05$) wider band of cell death at the wound edges of tissue punches digested with Chondroitinase AC (1U/ml, 15min) compared to undigested controls (Fig VI-4). At 7 days post-wounding, 3 out of the 4 samples showed a significantly ($p<0.05$) wider band of cell death at the GAG-digested wound edge compared to undigested controls. The widths of the bands of cell death seen at the experimental wound edges were approximately the same 1-day post-wounding as at 7 days post wounding (GAG-digested samples and controls, Fig VI-4).

GAG-digestion performed on the wound edge after it has been cultured for 1 or 7 days post-wounding (Figure VI-5)

For tissue samples subjected to GAG-digestion post-wounding and post-culture, 3 out of 4 samples showed a significantly ($p<0.05$) wider band of cell death at the wound edge digested with Chondroitinase AC (1U/ml, 15min) compared to undigested controls. This was evident at both 1 and 7 days post-wounding (Fig VI-5). The widths of the bands of cell death seen at the experimental wound edges tended to increase over time from 1-day to 7 days post wounding (also observed for undigested controls, Fig VI-5)

The difference between digested and undigested samples was greatest for tissue punches subjected to GAG-digestion immediately post-wounding and prior to culture (Figure VI-4) as opposed to being digested following wounding *and* culture (Figure VI-5). This treatment had the most significant effect in increasing the loss of cell viability at the experimental wound edges.

Figure VI-4 Width of the band of cell death seen at experimental wound edges in articular cartilage explants cultured over 7 days post-wounding – effect of digestion of glycosaminoglycans performed post-wounding but prior to culture

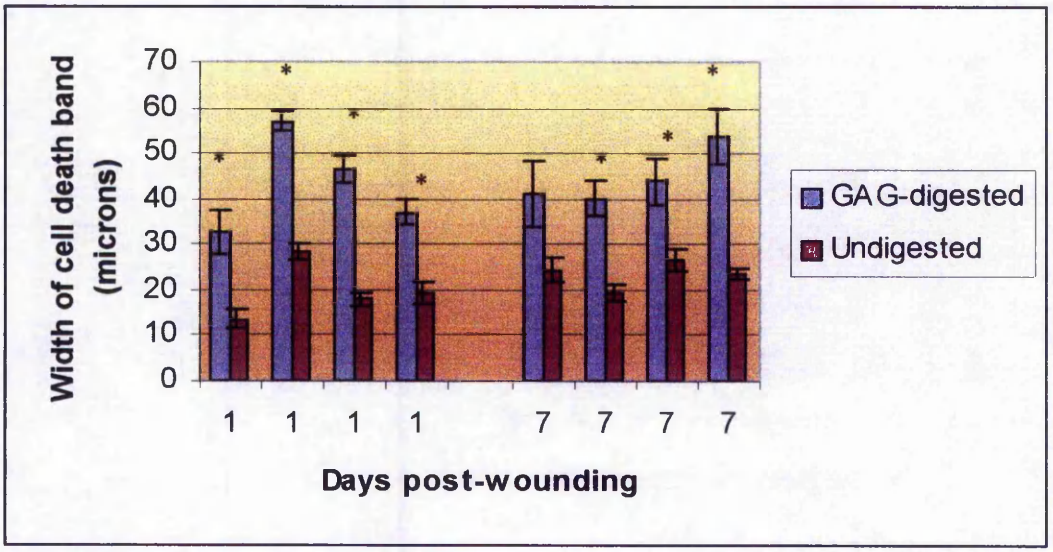

Figure VI-4

Articular cartilage explants were wounded twice and the tissue punches removed. One tissue punch was digested with Chondroitinase AC (1U/ml) for 15 minutes at 37°C prior to culture, whilst the undigested control tissue punch received 50mM Tris.Cl buffer (pH 7.3) in place of CAC solution. Both tissue punches were then cultured in standard media at 37°C, 5% CO_2 for 1 and 7 days post-wounding. Four explants were harvested at each time point and the widths of the bands of cell death at each wound edge were measured and compared according to the method described in VI-2.3 (figures show average of 8 measurements ± standard error of the mean). Statistically significant data (digested compared to undigested) are marked as * ($p<0.05$).

Figure VI-5 Width of the band of cell death seen at experimental wound edges in articular cartilage explants cultured over 7 days post-wounding – effect of digestion of glycosaminoglycans performed after wounding and culture

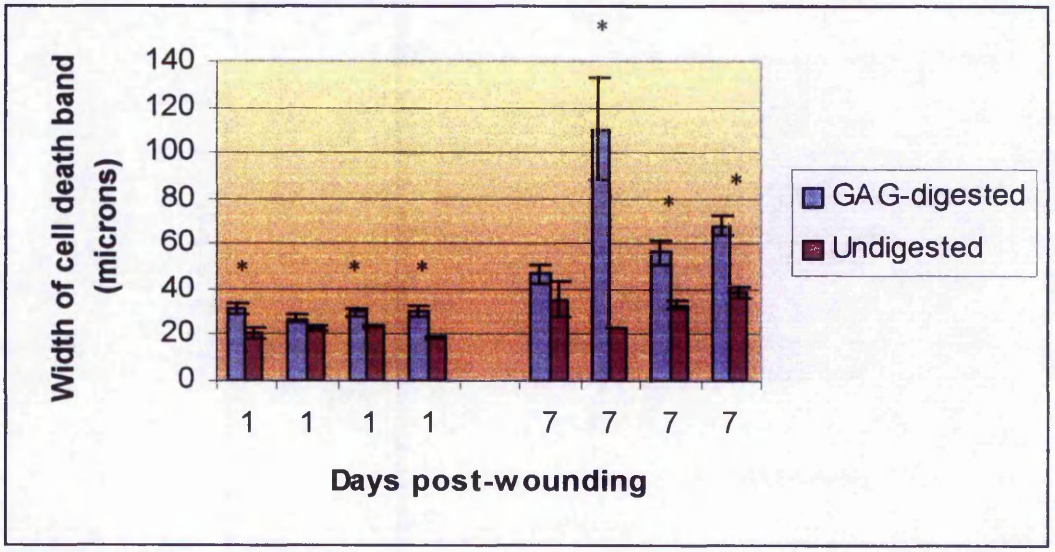

Figure VI-5.

Articular cartilage explants were wounded twice and both punches were removed and cultured in standard media at 37°C, 5% CO_2 for 1 and 7 days post-wounding. Prior to viewing, one tissue punch was digested with Chondroitinase AC (1U/ml) for 15 minutes at 37°C whilst the undigested control tissue punch received 50mM Tris.Cl buffer (pH 7.3) in place of CAC solution. Four explants were harvested at each time point and the widths of the bands of cell death at each wound edge were measured and compared according to the method described in VI-2.3 (figures show average of 8 measurements ± standard error of the mean). Statistically significant data (digested compared to undigested) are marked as * ($p<0.05$).

VI-3.1.4 Effect of oxygen concentration

The loss of cell viability at an articular cartilage wound edge did not seem to be influenced by alterations in the oxygen levels in culture (Fig VI-6).

Whilst at 1 day post-wounding, 2 out of 4 samples showed a statistically significantly ($p<0.05$) wider band of cell death at wound edges cultured at 5% oxygen compared to their controls cultured at 20% oxygen (Fig VI-6), the difference was small.

And in contrast, at 7 days post-wounding one out of three samples had a significantly ($p<0.05$) wider band of cell death at a wound edge cultured at 20% oxygen, compared to the tissue punch cultured at the lower 5% oxygen concentration (Fig VI-6).

These results do not suggest a conclusive effect of the oxygen levels in culture, on cell viability at articular cartilage wound edges.

VI-3.1.5 Effect of the presence of a caspase inhibitor

The loss of cell viability at an articular cartilage wound edge was not influenced by the presence of a general caspase inhibitor (z-FAD-FMK) in the culture medium (Fig VI-7).

For all samples at both 1 and 7 days post-wounding, the width of the band of cell death was not significantly different at wound edges cultured in the presence of the inhibitor, compared to control tissue punches cultured in its absence (Fig VI-7).

For both sets of tissue punches (with and without caspase inhibitor), the widths of the bands of cell death seen at the experimental wound edges tended to increase over time (7 days post wounding compared to 1-day post-wounding).

Figure VI-6 Width of the band of cell death seen at experimental wound edges in articular cartilage explants cultured over 7 days post-wounding – effect of oxygen levels in culture

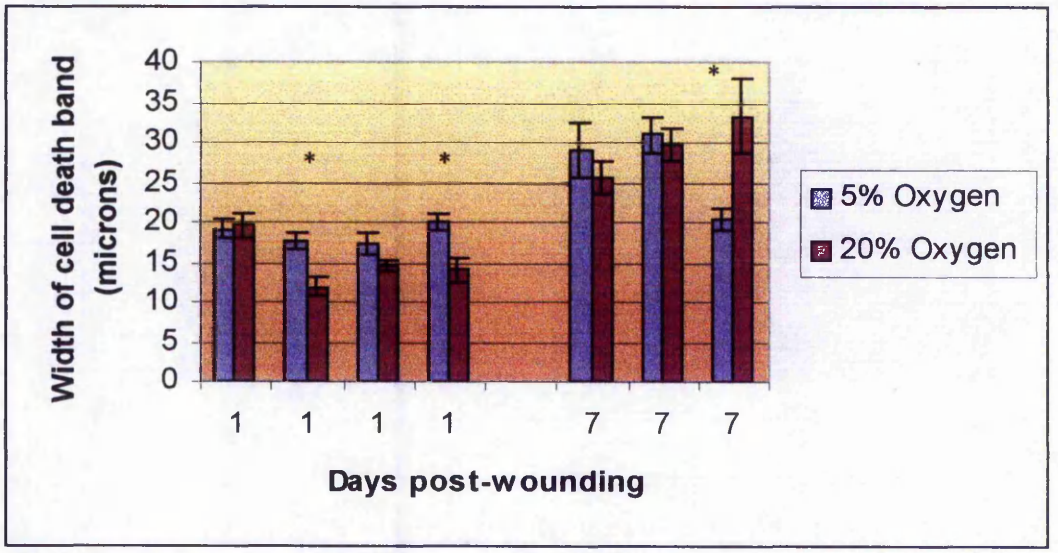

Figure VI-6.

Articular cartilage explants were wounded twice and the tissue punches removed. One tissue punch was cultured in standard media at 37°C, 5% O_2, 5% CO_2 for 1 and 7 days post-wounding. The control punch was cultured in standard media under standard conditions (37°C, 20% O_2, 5% CO_2) for 1 and 7 days post-wounding. Four explants were harvested at 1-day post-wounding and three at 7-days post-wounding. The widths of the bands of cell death at each wound edge were measured and compared according to the method described in VI-2.3 (figures show average of 8 measurements ± standard error of the mean). Statistically significant data (20% oxygen compared to 5% oxygen) are marked as * ($p<0.05$).

Figure VI-7 Width of the band of cell death seen at experimental wound edges in articular cartilage explants cultured over 7 days post-wounding – effect of the presence of a caspase inhibitor

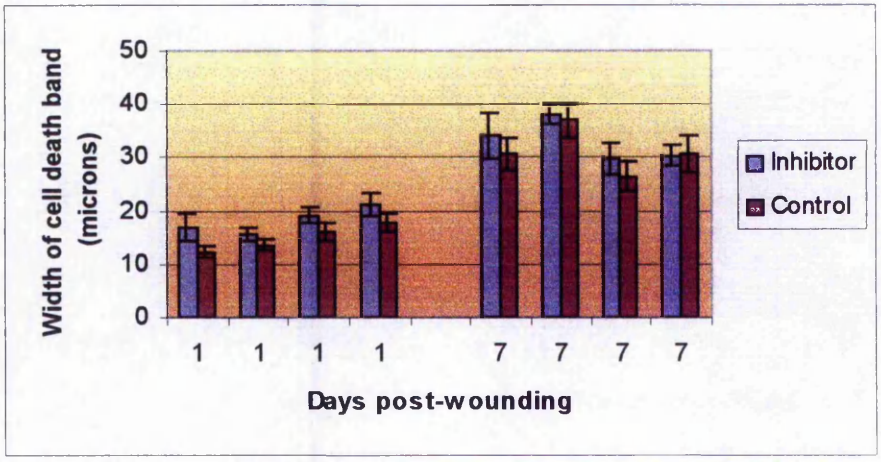

Figure VI-7.

Articular cartilage explants were wounded twice and the tissue punches removed. One tissue punch was cultured in standard media containing 20μM z-FAD-FMK caspase inhibitor at 37°C, 5% CO_2 for 1 and 7 days post-wounding. The control punch was cultured in standard media under standard conditions for 1 and 7 days post-wounding. Four explants were harvested at each time-point and the widths of the bands of cell death at each wound edge were measured and compared according to the method described in VI-2.3 (figures show average of 8 measurements ± standard error of the mean). Statistically significant data (compared to controls without inhibitor) are marked as * ($p<0.05$).

VI-3.1.6 Effect of tTG-catalysed bonding at the wound edge

The level of cell viability at an experimental articular cartilage wound edge over time was not influenced by tTG-catalysed bonding at the wound edge.

Tissue punches were incubated in tTG solution (4mg/ml containing 50mM EDTA to reduce premature activation) or buffer control solution, for 20 minutes at 37°C post-wounding and replaced within their respective explants for culture. With the intention of binding tTG to the cut wound edge, which would then become activated and cross-link the tissue interface once the punch was replaced into the explant and exposed to calcium in the culture media.

At 1 day post-wounding, no significant differences were observed between the widths of the bands of cell death seen at the tTG-bonded wound edges compared to their controls (Fig VI-8).

At 7 days post-wounding, one out of the three samples did show a significantly ($p<0.05$) wider band of cell-death at its tTG-bonded wound edge compared to its control (Fig VI-8).

tTG-catalysed bonding of a wound edge did not appear to lead to definite significant changes in cell viability at the wound edge compared to controls.

For both sets of tissue punches (tTG-bonded and controls), the widths of the bands of cell death seen at the experimental wound edges tended to increase over time (7 days post wounding compared to 1-day post-wounding).

Figure VI-8 Width of the band of cell death seen at experimental wound edges in articular cartilage explants cultured over 7 days post-wounding – effect of tTG-catalysed bonding at the wound edge

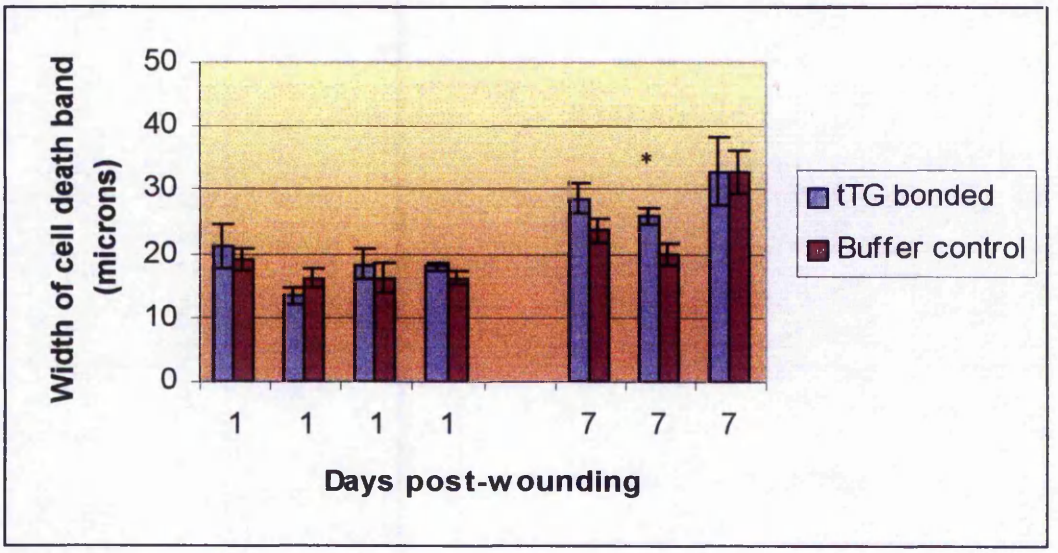

Figure VI-8.

Articular cartilage explants were wounded twice and the tissue punches removed. One tissue punch was incubated in tTG solution (4mg/ml containing 50mM EDTA) for 20 minutes at 37°C, before being replaced back into the surrounding explant tissue. The control punch was incubated in 50mM Tris.Cl buffer (pH 7.3) in place of tTG solution. The explants were then cultured under standard conditions for up to seven days post-wounding. Four explants were harvested at 1-day post-wounding and three at 7 days post-wounding. The widths of the bands of cell death at each wound edge were measured and compared according to the method described in VI-2.3 (figures show average of 8 measurements ± standard error of the mean). Statistically significant data (tTG-bonded compared to buffer control) are marked as * ($p<0.05$).

VI-3.2 Estimating the degree of TG activity at the surface of articular cartilage sections

It was shown previously that tTG-catalysed bonding, at an experimental wound edge in articular cartilage explants, had no significant effect on the loss of cell viability seen at the wound edge over time (compared to controls, see VI-3.1.6).

This bonding was achieved by incubating cartilage tissue punches in tTG solution (4 mg/ml containing 50mM EDTA for 20 minutes at 37°C, or 50mM Tris.Cl buffer (pH 7.3) for controls) before replacing them back into their host explant and culturing the tissue in media. The calcium present in the media should then activate the tTG that became bound to the wound edge, causing it to catalyse cross-linking at the interface between the wound edge and its surrounding explant tissue.

In order to determine if tTG could be bound to cut edges of articular cartilage in this way, sections of articular cartilage explant were incubated with either tTG solution (1 and 4mg/ml containing 50mM EDTA) or buffer control for 20 minutes at 37°C, followed by incubation with standard media containing Texas-Red cadaverine (for method see VI-2.5).

An increased incorporation of Texas-Red cadaverine into the tissue section would then be taken to indicate an increased level of TG activity on the section surface.

Figure VI-9 shows the degree of incorporation of Texas-Red cadaverine (TC) into articular cartilage section surfaces, with and without their pre-incubation with tTG solution (as analysed using CLSM).

Increased incorporation of TC was shown into those sections that were previously incubated with tTG solution, compared to that which received buffer control in place of tTG (Fig VI-9A & C compared to Fig VI-9E). This increase was not seen in the positive controls where TC incorporation was performed in the presence of EDTA (see FigVI-9B, D & F), indicating that the increased incorporation is due to a higher level of TG activity on these surfaces.

This infers that incubating articular cartilage sections in tTG solution causes tTG to become bound to the surface of the tissue section, which is then activated in the

presence of the calcium contained in standard media, resulting in the increased incorporation of Texas-Red cadaverine into the section surface.

Therefore incubating articular cartilage tissue punches in tTG solution should also cause tTG protein to become bound to the tissue surface (including the wound edge), which will then become activated when placed back into the articular cartilage explant and cultured in standard media. This activated tTG should then catalyse cross-linking at the interface between the wound edge and the surrounding explant tissue. Thus validating the method used previously to achieve this effect and measure subsequent levels of cell viability at the bonded wound edge (see VI-3.1.6).

The increase in incorporation of Texas-Red cadaverine showed a concentration dependence with respect to the tTG solution, with more TC was incorporated into the section which was pre-incubated with 4mg/ml tTG than into that which received 1mg/ml tTG (Fig VI-9C compared to Fig VI-9A).

As was found previously (see IV-3.5), a degree of endogenous TG activity was seen in the cartilage tissue. With some TC incorporation into the section which received buffer in place of tTG (Fig VI-9E).

Some TC incorporation was also seen in the positive controls (Fig VI-9 B, D & F) suggesting that the level of TG inhibitor used during the incorporation (20mM EDTA final) may not have inhibited all the TG activity in the tissue section.

Figure VI-9 *In situ* incorporation of Texas-Red cadaverine (TC) into articular cartilage sections, pre-coated with either tTG or buffer control

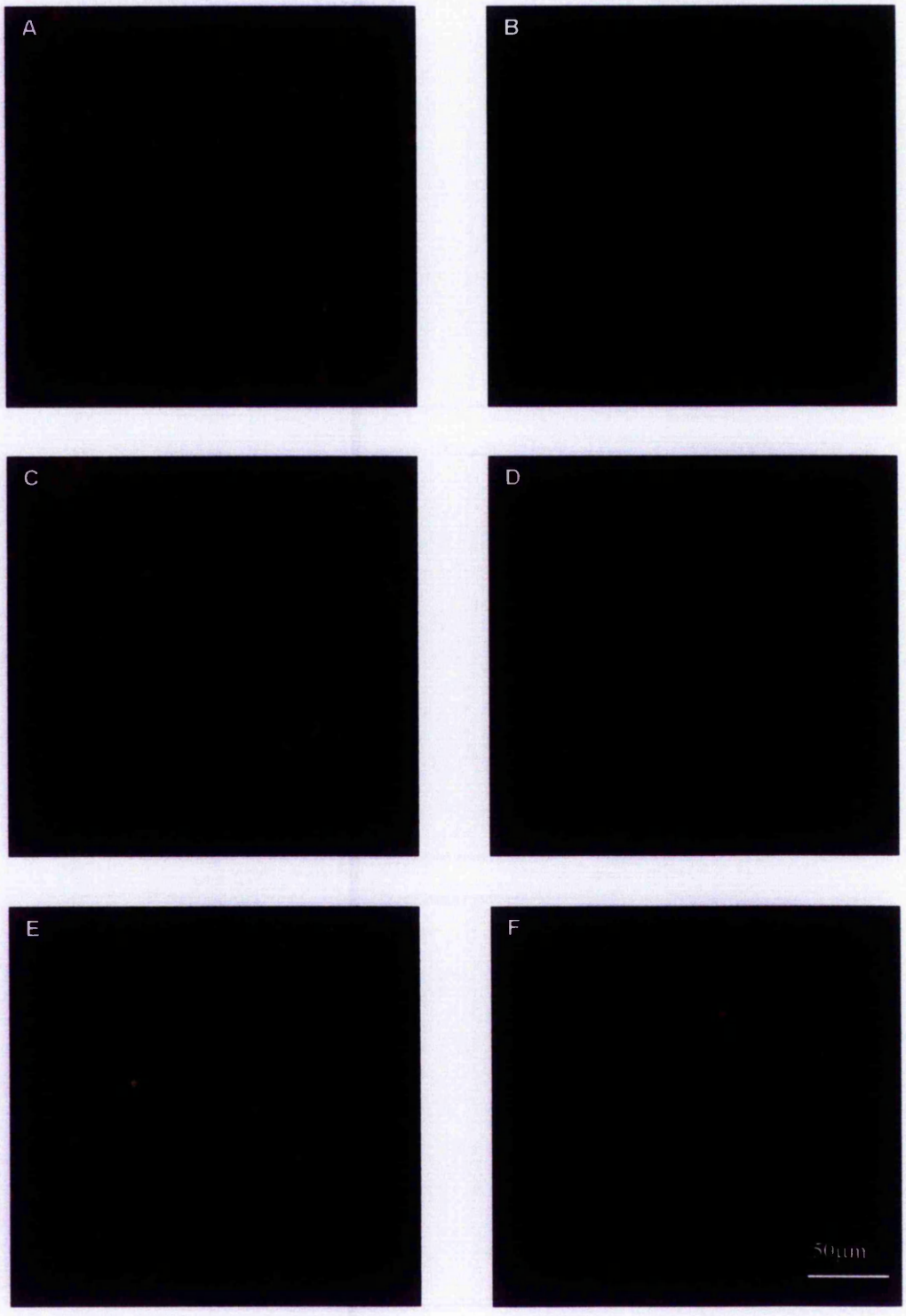

Figure VI-9.

Texas-Red cadaverine (TC) incorporation into articular cartilage sections, following incubation at 37°C for 30 minutes in standard media (see VI-2.5).

Tissue sections were pre-incubated before TC incorporation with 20µl of either tTG solution (1 and 4 mg/ml plus 50mM EDTA) or buffer control.

Sections then received standard media containing 5µm TC and were incubated at 37°C for 30 min. Positive controls for TG-catalysed incorporation received TC in standard media in the presence of 20mM EDTA.

Fig VI-9A TC incorporation into an articular cartilage section pre-incubated with tTG solution (1mg/ml)

Fig VI-9B TC incorporation (in the presence of EDTA) into an articular cartilage section pre-incubated with tTG solution (1mg/ml)

Fig VI-9C TC incorporation into an articular cartilage section pre-incubated with tTG solution (4mg/ml)

Fig VI-9D TC incorporation (in the presence of EDTA) into an articular cartilage section pre-incubated with tTG solution (4mg/ml)

Fig VI-9E TC incorporation into an articular cartilage section pre-incubated with buffer control

Fig VI-9F TC incorporation (in the presence of EDTA) into an articular cartilage section pre-incubated with buffer control

VI-3.3 Comparison of the level of cell death occurring at the various experimental wound edges

To compare the effects of the various conditions investigated, on cell viability at experimental articular cartilage wound edges, the width of the band of cell death measured at each treated wound edge was expressed as a % of that measured at the wound edge of its respective control tissue punch. In that, the distance that the band of dead cells had progressed into each treated wound edge was expressed as a % of that measured at its untreated control wound edge and this data averaged for each explant set, for each treatment investigated, at both 1 and 7 days post-wounding. Figure VI-10 shows the width of the band of cell death occurring at treated experimental wound edges in articular cartilage explants (cultured over time post-wounding) expressed as a % of that measured at untreated control wound edges.

Culturing the wound edge in the presence of serum, or in contact with the surrounding explant tissue, both had the effect of reducing the level of cell death seen at the wound edge. This decrease was statistically significant for both time-points in the case of contact with the surrounding explant, and for 7 days post-wounding in the case of the presence of serum in the culture media. The biggest decrease was shown at wound edges cultured in the presence of serum after 7 days post-wounding, compared to controls cultured in serum-free media.

Digestion of GAG's at the wound edge, whether done immediately after wounding and prior to culture or done after both wounding and culture, significantly increased the level of cell death occurring at the wound edge at both 1 and 7 days post-wounding.

Culturing the wound edge in reduced oxygen conditions or after bonding the wound edge to the surrounding explant tissue via tTG-catalysed bonding, both had no significant effect on the level of cell death measured at the wound edge. At 1 and 7 days post-wounding, the presence of tTG-catalysed bonding led to slightly higher but insignificant levels of cell death compared to controls. Culturing wound edges in reduced oxygen conditions (5% O_2 compared to 20%O_2) led to a slight increase in the level of cell death seen at the wound edge at 1 day post-wounding. And at 7 days post-

wounding the effect was a slight reduction in the level of cell death, but neither of these changes were statistically significant.

Culturing the wound edge in the presence of caspase inhibitor led to a small, significant increase in cell death at the wound edge after 1-days culture post-wounding. After 7 days, this increase was even less and not statistically significant.

Figure VI-10 Width of the band of cell death seen at treated experimental wound edges in articular cartilage explants cultured over 7 days post-wounding – expressed as a % of untreated controls

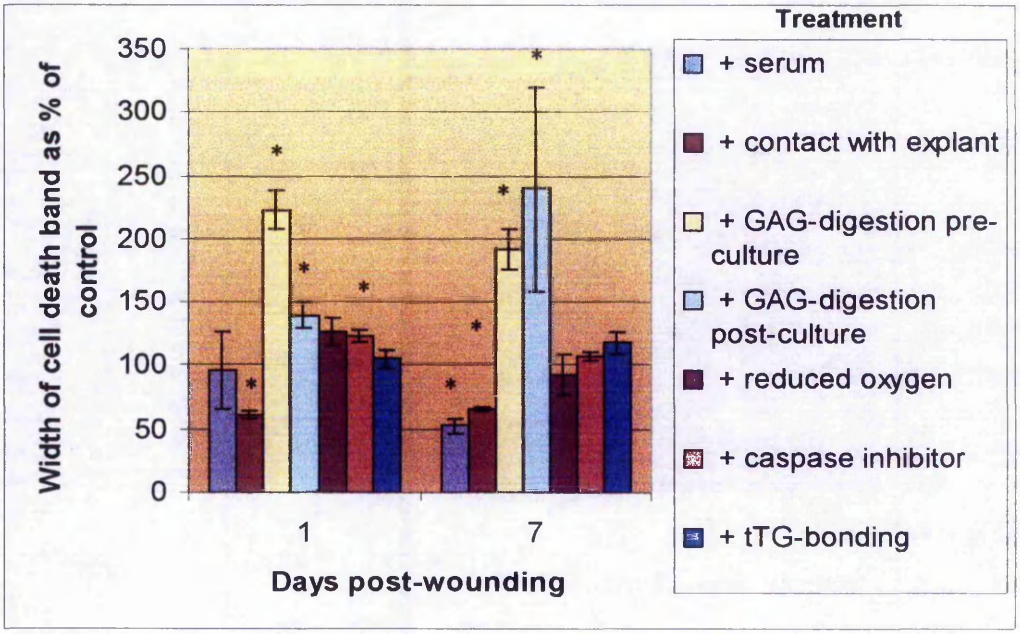

Figure VI-10 Articular cartilage explants were wounded twice and one tissue punch treated to determine the effects of the treatment on cell viability at the resulting experimental wound edge. The second tissue punch was used as a control, left untreated and cultured under standard conditions (for details of treatment methods and nature of untreated controls see VI-2.4). Explants were then cultured for up to seven days post-wounding and harvested at 1 and 7 days post-wounding. The widths of the bands of cell death at each wound edge were measured according to the method described in VI-2.3. The width of the cell-death band at the wound edge of each treated punch was then expressed as a % of that of its respective control and the data for each treatment averaged for each time-point (figure shows average of either 3 or 4 explants ± standard error of the mean). Statistically significant data (compared to untreated controls) are marked as * ($p<0.05$).

VI-IV Conclusions

An *in vitro* articular cartilage explant culture system was used to measure the degree of cell death occurring over time, at experimental wound edges made within the tissue.

Loss of chondrocyte viability has been shown to occur at the margins of defects in cartilage tissue (Mankin, 1962; Shapiro *et al*, 1993; Tew *et al*, 2000). A characteristic band of dead cells forms around the edges of the lesion, which can progress into the surrounding tissue. This response of cartilage to wounding may have serious detrimental effects on cartilage repair efforts. The fact that the tissue at the wound edge is non-viable may be hindering integration between repair materials / tissue and the recipient host cartilage. Even if integration is initially achieved, the fact that any newly formed repair tissue is merely bound to a region of dead cells may then cause long-term repair to fail as the two areas do not go on to form a continuos region of healthy tissue.

In the explant culture system developed, wounding was done using a biopsy punch and the degree of cell death occurring at the resulting wound edge was assessed using a two-colour fluorescence cell viability stain and confocal laser scanning microscopy. The level of cell death occurring at each wound edge was quantitated by image analysis and measuring the distance that the band of cell death had progressed from the wound edge, toward the centre of the circular tissue punch sample. Thus for each tissue punch, the width of the band of cell death seen around its circumferential wound edge was recorded and compared to that seen at the wound edge of a control punch, taken from the same tissue explant.

The loss of cell viability at an articular cartilage wound edge was reduced when the tissue was cultured in the presence of foetal calf serum. The effect was more evident over time, with 3 out of 4 samples at 7 days post-wounding showing significantly ($p<0.05$) wider bands of cell death at wound edges cultured in the serum-free media compared to controls cultured in the presence of 10% FCS. For 1-day post wounding 2 out of 4 samples showed such a difference.

This loss of cell viability after culture in serum-free conditions showed that the experimental model used (and quantitation method) was capable of highlighting differences in the level of cell death occurring at articular cartilage wound edges. It also suggests that factors contained within the serum promoted chondrocyte survival at the wound edge.

The degree of cell death seen at experimental articular cartilage wound edges was also reduced when the tissue punch remained in contact with the surrounding explant tissue. For all samples at 1, 5 and 7 days post-wounding, the cell death band at the wound edge was significantly ($p<0.05$) wider for control tissue punches removed to media, compared to those that were cultured in contact with the surrounding explant tissue.

This suggests that the presence of neighbouring chondrocytes or extra-cellular matrix may provide some kind of survival stimuli for cells, which is then lost when the tissue punch is removed from its explant. Thereby leading to the increased levels of cell death seen at the wound edges of removed samples. This increase in cell death occurred after only 1-days culture of the wound edge without contact with surrounding tissue. Similar results have been reported in previous studies of chondrocyte viability at experimental wound edges *in vitro* (Thompson, 1999) and a loss of extra-cellular matrix survival signals has been suggested as an inducer of apoptosis in articular chondrocytes (Hashimoto *et al*, 1998). One implication of this finding is that if cartilage tissue is harvested for the purpose of transplant (e.g. chondral auto / allografts), then it should only be removed immediately prior to implantation. In order that chondrocyte viability at the edges of the graft is maximised, which may then help to promote integration between the implanted and native tissues, improving the prospects for long-term cartilage repair.

If close contact between the wound edges of a cartilage-cartilage interface helps to reduce the loss of cell viability seen at the tissue edges over time, then cross-linking the two interface surfaces together using a tTG-based adhesive may enhance the effect. Application of tTG to a cartilage-cartilage interface may not only help to stabilise the interface, but also ensure even greater contact and integration between the

tissue surfaces through its cross-linking of cartilage extracellular-matrix proteins. Hence, possibly further enhancing the improvement in cell viability levels seen at cartilage wound edges cultured in contact with surrounding tissue, compared to controls cultured without such contact.

The level of cell death occurring at experimental wound edges pre-coated with tTG and subsequently cultured in contact with surrounding tissue was not found to differ from wound edges incubated with a buffer control solution and cultured in contact with cartilage explant tissue. Incubating tissue punches in a tTG solution (4mg/ml for 20min at 37°C), before replacing them for culture within tissue explants, did not improve levels of cell viability measured at the cartilage wound edge, when compared to controls also cultured in contact with surrounding tissue but which received buffer in place of tTG.

In order to determine if tTG was becoming bound to the cartilage wound edges using this incubation method, sections of articular cartilage explant were incubated in a similar fashion (tTG solution (1 & 4 mg/ml) or buffer control added for 20 minutes at 37°C). The sections were then incubated in standard media containing Texas-Red cadaverine (TC), with an increased incorporation of TC into the tissue being taken to indicate an increased level of TG activity on the section surface.

Results showed that prior incubation of articular cartilage sections with tTG solution led to an increase in the incorporation of TC into those sections (compared to controls incubated with buffer solution). This increase was not shown in the presence of EDTA, which indicates that the increase was due to enhanced TG activity on the section surface. Although a small degree of TG activity was still evident on the section surface in the presence of EDTA, whose concentration (20mM) may not have been high enough to inhibit all TG activity within the tissue section. However, these results suggest that incubation of tissue sections with tTG solution caused tTG protein to become bound to the section surface, which was then activated by calcium levels present in the standard media. It may be inferred therefore that the same would occur during the incubation of articular cartilage tissue punches with tTG solution. Suggesting this incubation to be a valid method of applying tTG to the wound edge of a cartilage tissue punch, which then becomes activated when the punch is replaced within a cartilage explant and cultured in media containing Ca^{2+}.

Whilst the application of tTG to cartilage wound edges did not appear to have a beneficial effect, in terms of levels of cell viability at the tissue margins, it also did not have a negative effect.

Aside from one sample at 7 days post-wounding, the application of tTG led to no significant decreases in levels of chondrocyte viability at cartilage wound edges. This would seem to be an indication that the application of tTG to bond cartilage-cartilage interfaces during *in vivo* repair would not be detrimental to the tissues with regard to cell viability. If it had, then the enzymes application, whilst stabilising the tissue interface, may have had detrimental effects on the prospects for fully restoring tissue structure and function. Adhesive strength between bovine articular cartilage explants maintained in apposition in an *in vitro* culture system, has been shown to be dependent on the number of viable cells within the tissues (Reindel *et al*, 1995).

The apparent failure of tTG-catalysed bonding to reduce the loss of cell viability seen at cartilage wound edges may also have been due to not enough tTG protein being present to cross-link the wound edge interface effectively. As the increase in TG activity seen in tissue sections incubated with tTG was not great, suggesting the amount of tTG protein bound to the tissue surface to be small.

A treatment method that has been proposed for aiding cartilage repair that did have a detrimental effect on chondrocyte viability was the digestion of glycosaminoglycans.

GAG-digestion has been proposed as a method for promoting the attachment of repair materials to cartilage surfaces (Hunziker & Rosenberg, 1996) and aiding tTG catalysed bonding of cartilage-cartilage interfaces (see III-3.5.4 and Jurgensen *et al*, 1997). However, the digestion of GAG's at experimental wound edges was shown to lead to an increased loss of cell viability.

The digestion of GAG's (CAC 1U/ml for 15 minutes at 37°C) had an immediate effect on cell viability levels. When the digestion was performed immediately prior to the assay of cell viability (i.e. after both wounding and culture of the tissue punches), three out of four digested samples showed significantly ($p<0.05$) wider bands of cell death compared to undigested controls, at both 1 and 7 days post-wounding.

A similar effect was also observed when the GAG-digestion preceded the period of culture. For tissue samples digested post-wounding (CAC 1U/ml for 15 minutes at 37°C) but prior to culture, all samples at 1-day post-wounding showed a significantly ($p<0.05$) wider band of cell death at the CAC digested edges compared to controls. After 7 days of culture, 3 out of 4 digested samples showed this increase.

This effect on cell viability may be attributable to a disruption of the extra-cellular matrix surrounding the chondrocytes, of which GAG's are an important part.

It was suggested earlier that chondrocyte viability might be influenced by a 'survival signal' emanating from surrounding matrix or cells. And that the disruption of this signal through mechanical wounding of the tissue leads to a loss of chondrocyte viability at the wound edge. The digestion of GAG's at cartilage wound edges may have caused increased disruption of the extra-cellular matrix at the tissue margins, and either disrupted matrix deeper into the tissue from the wound edge or enhanced the loss of the 'survival signal' as felt by chondrocytes at the wound edge. This may then have led to the increased width of the band of cell death seen at the GAG-digested wound edges compared to undigested controls. So whilst GAG-digestion may enhance the attachment of repair materials to cartilage surfaces, it seems to increase the loss of chondrocyte viability at the wound surface. And its action may merely aid the repair materials to attach to a wider band of dead tissue that would be found at undigested surfaces, an outcome which may have detrimental effects on the prospects for repair.

The loss of cell viability at an articular cartilage wound edge was not influenced by alterations in the oxygen levels during culture after wounding.

As cartilage is an avascular tissue, the *in vivo* oxygen tension in the tissue is low (approximately 1-7 %). It has been suggested that the proliferative response of cultured chondrocytes *in vitro* can differ when exposed to varying oxygen tensions (Cooling *et al*, 2000) and also that the de-differentiation of the chondroyte phenotype observed in culture may be controlled by varying the oxygen levels (Murphy & Sambanis, 2000). These findings have implications for the long-term culture of cartilage and chondrocytes and the successful growth of tissue engineered cartilage in bioreactors.

Our results shows that experimental wound edges within articular cartilage explants show no significant reduction in the loss of cell viability seen at the wound edge, when cultured at 5% O_2 compared to controls cultured in conditions of 20% O_2. However, whilst the tissue was pre-incubated at 5% O_2 prior to wounding and rapidly replaced to this environment afterwards, the cutting itself took place in atmospheric conditions. So immediately following wounding the cells at the wound edge were not in a low oxygen environment.

Whilst the cell death seen at cut cartilage wound edges has been attributed largely to necrosis (Mankin *et al*, 1962) it has also been suggested that some of the cells may be dying by a process of apoptosis (Tew *et al*, 2000).

In this study, attempts to determine the form of cell death occurring at the experimental wound edges were not made. However should apoptosis be a contributory factor, preliminary studies were made to determine the degree of cell death occurring at experimental wound edges, measured in response to the presence of a general caspase inhibitor.

Activation of caspases during apoptosis results in the cleavage of critical cellular substrates (Cohen, 1997). Cleavage of these substrates, such as structural elements of the cytoplasm and nucleus, components of the DNA repair machinery, and a number of protein kinases, contributes to the characteristic morphological and biochemical changes that characterise apoptotic cell death (Earnshaw *et al*, 1999).

Z-VAD-FMK is a cell-permeable inhibitor of caspase, but its presence in culture did not reduce the loss of cell viability seen at experimental articular cartilage wound edges in this study. The width of the band of cell death measured at wound edges of tissue punches cultured in the presence of 20μM Z-VAD-FMK was not significantly different to those of the control samples, at both 1 and 7 days post-wounding. It is possible that the inhibitor may not have been present in an effective amount to penetrate into the tissue and was only exposed to chondrocytes at the immediate tissue margins. Although 20μM Z-VAD-FMK has been shown to be capable of inhibiting Fas mAB induced apoptosis in Jurkat cells (product literature).

These results would suggest that either caspase-induced apoptosis is not a significant contributor to the cell death occurring at cut articular cartilage wound edges, or that the reduced cell death its inhibition may bring is not detectable using this system.

In order to compare the levels of chondrocyte death seen at experimental cut cartilage wound edges, in response to the variables investigated, the width of the band of cell death seen at treated wound edges was expressed as a % of that seen at the wound edges of control tissue punches. This data was then averaged for each set of cartilage explants and compared.

A significant ($p<0.05$) reduction in cell death was seen at wound edges cultured for 7 days post-wounding in the presence of 10% FCS (compared to serum-free conditions). And also at wound edges cultured for both 1 and 7 days post-wounding in contact with surrounding explant tissue (compared to controls cultured without contact). For each of these three instances, the width of the band of cell death at treated wound edges fell to approximately 50-60% of control levels

GAG-digestion led to a significant ($p<0.05$) increase in cell death at wound edges at all time-points post-wounding. With the greatest increase seen at edges digested following both wounding and 7-days of culture. The width of the cell death bands at GAG-digested wound edges was approximately 200% of control levels for edges digested *immediately* following wounding and *prior* to culture for both 1 and 7 days. For samples digested *following* a period of post-wounding culture, the width of the band of cell death at digested wound edges was around 130% of controls after 1-days culture and approximately 250% after 7 days.

The other variables investigated (presence of capsase inhibitor, presence of tTG-catalysed bonding or reduced oxygen levels during culture) had no significant effect on the width of the band of cell death seen at treated wound edges compared to controls. Except for the presence of caspase inhibitor at 1 day post-wounding, which saw a small increase in cell death to around 125% of that of the control.

Chapter VII : General discussion

Chapter VII : General Discussion

Chapter VII-1 Discussion

The general aim of this work was to investigate the potential of Transglutaminases, in particular tissue transglutaminase (tTG), to act as tissue bonding agents with focus on its application in the field of cartilage repair.

The intrinsic repair of cartilage defects is rarely successful in the long term due to factors that limit its response to injury (Buckwalter *et al*, 1988; Hunziker, 1999a). Various cartilage repair technologies have been developed (see I-3.4), but a clinical limitation of these has been the secure anchoring or attachment of repair materials into defect sites. These materials might comprise combinations of cells, tissues (such as cartilage, bone or periosteum), or synthetic and biological matrices. Whichever method and material is used, successful repair requires the integration of the repair tissue with host cartilage and this integration is often not forthcoming (Hunziker, 1999a; Grande *et al*, 1999; Temenoff & Mikos, 2000). This has prompted investigation into the development of a successful tissue adhesive for use in cartilage repair.

In this study an *in vitro* test method was developed to measure bond strength at cartilage-cartilage interfaces in response to tensile stress.

Integrative repair of opposing cartilage surfaces is required for the fissures that appear in cartilage during osteoarthritis, as well as following blunt trauma, chondral and osteochondral fracture and surgical procedures of grafting (Ahsan & Sah, 1999).

The bond strength of tissue transglutaminase (tTG) preparations, at interfaces between the cut surfaces of porcine hyaline cartilage, was found to increase with increasing enzyme concentration. Tissue transglutaminase plays an active role in the adhesion process as the addition of a TG inhibitor (EDTA) to the enzyme preparation reduced its bond strength to levels shown by the buffer control solution. The relationship of bond strength to tTG concentration was also found for bonded cartilage-cartilage interfaces subjected to shear stress (Jurgensen *et al*, 1997). Failure of the commercial tTG to re-constitute at concentrations higher than 8mg/ml prevented high concentration preparations being investigated, but the use of such a solution should produce an even greater bonding strength.

In practical terms such a product may have a high cost unless tTG can be produced relatively cheaply on a large-scale, possibly by recombinant means.

The production of recombinant TG has been proposed in several applications. Human and murine tTG were suggested for expression by recombinant means, preferably in cultured eukaryotic cells, which would allow for large scale production of tTG in relatively pure form (Davies & Stein, 1992). Since the sequence of the gene(s) is already known, fragments of the tTG polypeptide may be produced with TG activity present, or not, as desired. A cDNA, and tTG protein encoded thereby, have been obtained from reverse transcription of mRNA from retinoic acid-treated HEL cells, which has been used for recombinant tTG production (Fraij *et al*, 1998). The purified human tTG produced in this instance was smaller than isolated human cellular tTG, and had a molecular weight of 63 Kda. Though it still retained protein cross-linking activity. The application of this recombinant technology is not limited merely to the tissue form of the enzyme. It may be used to produce other members of the TG family as desired, such as prostate and placental transglutaminases, for use in various applications including food binding and skin graft closure (O'Hara *et al*, 1996).

Using the novel test system developed in this project, the bond strength of tTG was compared to that of a commercially available fibrin sealant (Beriplast®). At a concentration of 8mg/ml the tTG preparation showed a comparable bond strength to that of the commercial sealant under equivalent incubation conditions.

Increasing the duration of incubation led to an increase in the bond strength of both adhesives. This phenomenon was not observed in the buffer control which suggests that the greater bonding of tTG after longer incubation times was not merely due to the sample drying out and becoming tacky along the tissue interface. Given longer incubation times, a lower concentration tTG preparation reached the same level of bond strength as found for the higher concentration solution after a shorter incubation time. But the bond strength of both preparations appeared to be reaching a plateau after incubation for forty minutes, suggesting that tTG protein concentration and/or substrate numbers are important for determining bond strength, as well as enzyme activity.

Tissue transglutaminase can itself act as a glutaminyl substrate, catalysing rapid, auto-catalytic cross-linking. Immunostaining for tTG along a cartilage-cartilage interface bonded by the enzyme was found to be restricted to the interface, with no

penetration into the tissue (Jurgensen *et al*, 1997). Auto-catalytic cross-linking of the enzyme onto the tissue surfaces was demonstrated and this is likely to be occurring at the cartilage-cartilage interfaces investigated using the novel test method developed.

How practical longer incubation times would be to the surgical user remains to be seen. A disadvantage of fibrin sealant is that its' relatively low bonding strength means that when applied in orthopaedic situations, the joint often needs to be immobilised following application (Claes *et al*, 1980). It would be of benefit if the bond strength of tTG could be great enough, rapidly enough, to prevent this from being necessary following tTG application also.

Tissue transglutaminase was also shown to maintain its bonding capacity in high humidity incubation conditions, indicating that the enzyme should maintain its gluing capacity if applied in the fluid-filled atmosphere of the body. This is vital for a tissue adhesive as even if water is removed from the tissue surface prior to adhesive application, water beneath can migrate onto the bonding surface and effect the curing process (Ikada, 1997). In contrast, the commercial fibrin sealant saw a significant reduction in its' bond strength following incubation at 90-100% humidity.

Stress-strain curves (plots of force vs. displacement), obtained from measuring bond strength in response to tensile stress at cartilage-cartilage interfaces, showed an approximately linear increase to the point of failure for both tTG and Beriplast®. An initial lag period was observed for both preparations, but otherwise the plots were as observed in previous studies of tTG and fibrin sealant bond strength at cartilage interfaces in response to shear stress (Jurgenesen *et al* 1997; Orr *et al*, 1999). Except that the plot obtained following Beriplast® application showed a sharper decline following maximum load than found for the fibrin sealants used in previous studies.

The overall trends in the response of tTG-catalysed bonding to tensile stress were also observed for tTG-catalysed bonding in response to shear stresses at cartilage-cartilage interfaces, in terms of incubation time, enzyme concentration and incubation humidity (Jurgensen *et al*, 1997). Comparison of the bond strength achieved in the two studies is difficult as, is often found in tissue adhesive investigations, the test methods differ and different stresses are being put on the bonded interface. However, in response to

shear stress tTG (1mg/ml) gave a bond strength of around 0.025 MPa after incubation for 10 minutes at 37°C, 50% humidity (Jurgensen *et al*, 1997). In this study, in response to tensile stress, tTG (2mg/ml) produced a bonding strength of around 0.029 MPa after incubation for 20min at 37°C, 20-30% humidity. After a 10-minute incubation under the same conditions, tTG at 4mg/ml also produced a bond strength of around 0.029 MPa. Whilst tTG seems to produce greater bond strength in response to shear stress (as opposed to tensile stress), these results indicate that the novel test method developed produces results that are consistent with the findings of others.

For both investigations into tTG-catalysed bonding at cartilage-cartilage interfaces, the enzyme preparation was shown to be capable of producing bond strengths that matched, and in some cases, exceeded those of a commercial fibrin sealant under equivalent conditions. Whilst this is encouraging, fibrin sealant bond strength is still considered too low to give proper wound approximation in application as a tissue adhesive (Claes *et al*, 1980; Spotzitz *et al*, 1997). However its other desirable properties of biocompatibility, biodegradation and quick application, may still apply for a tTG-based adhesive, whose use would also eliminate the use of blood-products currently necessary for fibrin sealant production.

The bond strength of tTG is still some way below those of the synthetic cyanoacrylates, though these suffer from problems of toxicity and biocompatibility when used *in vivo* (Ikada, 1997). In a comparative study of bonding in response to tensile stress at butt-jointed cartilage-cartilage interfaces, n-butyl-2-cyanoacrylate produced bond strengths of around 1.0 MPa, compared to 0.0049 MPa for a commercial fibrin sealant at equivalent interfaces (Chivers & Wolowacz, 1997). The bond strengths of tTG reported in this thesis exceed those reported for the fibrin sealant, but are below the bonding strength found for the synthetic adhesive.

Comparison of bond strengths between different investigations is difficult as most use varying test geometries, incubation conditions and adhesive compositions. The *in vitro* test method developed in this thesis allows for direct comparison of adhesives under equivalent test conditions. A comprehensive study of the bond strength of a variety of tissue adhesives such as fibrin sealants, gelatin-based adhesives, cyanoacrylates and tTG-based adhesives could then be performed at interfaces between a variety of tissues and materials. In order that the performance of each adhesive may be properly

judged against its alternatives and that the data regarding bonding of tissue repair materials to tissues may be considered during their design, as retention of such devices at the site of application is of prime importance if they are to facilitate tissue repair.

Efforts were made to increase the bond strength of tTG preparations by altering both the preparations themselves and the tissue surfaces to which they were applied. Since one of the features of cartilage is its anti-adhesive nature, attempts were made to aid tTG-catalysed bonding at cartilage-cartilage interfaces by pre-treating the tissue surfaces before enzyme application.

Physically scoring the tissue led to an increase in tTG bond strength that was not seen in the control solution. This indicates that the cutting of the interface may have been revealing more tTG-substrates on the tissue surfaces, which could increase the levels of TG-catalysed cross-linking at the interface, and raise bond strength. What effect this scoring may have had on cell viability at the cartilage surface remains to be seen. In studies of cartilage injury, a zone of cell death at the margins of lesions has been consistently noted, and it has been suggested that this may limit cartilage repair (Hashimoto *et al*, 1998b). Therefore creating further injury and lesions to the cartilage surface by scoring the tissue may promote bonding, but it may also increase the volume of dead tissue at the bonded interface and hinder repair in the long term.

This may also be the case for drying of the cartilage surfaces, a pre-treatment that raised the bond strength of both tTG and the buffer control solution. As cartilage is a tissue whose structure relies critically on its water/fluid content, drying the tissue is likely to lead to a loss of cell viability and tissue integrity at the surfaces of the dried interface.

Articular cartilage contains macromolecules that render its surface anti-adhesive, which hinders attempts at integration and bonding between the tissue and repair materials. This property is attributed to proteoglycans within the tissue (Temenoff & Mikos, 2000) and enzymatic digestion of their glycosaminoglycan (GAG) constituent has been shown to promote the attachment of repair materials to cartilage defect surfaces (Hunziker & Rosenberg, 1996) and enhance chondrocyte adhesion to cartilage surfaces (Lee *et al*, 1999). Removal of proteoglycans from the surface of articular cartilage defects *in vivo* has also been shown to transiently increase the coverage of the defect by 'repair' cells (showing characteristics of fibroblasts or

undifferentiated mesenchymnal connective-tissue cells, and macrophages) (Hunkizer & Kapfinger, 1998).

Using the novel test method reported in this project, pre-treatment of cartilage surfaces prior to bonding, via enzymatic digestion of GAG's, enhanced the bond strength of tTG at the tissue interface. The increase was time-dependent with respect to the duration of digestion and was not observed for the control solution. Various enzymes known to de-polymerise proteoglycans produced this effect, though application of several enzymes together had no synergistic effect on enhancing tTG bond strength (also found for tTG-bonding in response to shear stress (Jurgensen *et al*, 1997)).

GAG-digestion was combined with etching of the cartilage surfaces to see if these two pre-treatments, which were successful individually in raising tTG bond strength, could act synergistically. But a combination of the two appeared no more beneficial than one treatment alone, and as before, the physical scoring of the tissue is likely to have deleterious affects on chondrocyte viability at the interface surfaces.

Applying the GAG-digesting enzyme (Chondroitinase AC) whilst included in the tTG preparation, rather than as a pre-treatment, had the effect of reducing the bond strength of tTG. This was done to see if the time that would be required for GAG-digestion followed by tTG application could be reduced. But the two enzymes may not have given each other time to work, may have inhibited each other's action, the calcium-containing activating buffer may have inhibited the Chondroitinase AC, or CAC itself may have been cross-linked by tTG rendering it inactive.

Proteoglycan digestion is used to promote attachment of repair materials as it removes negative charge from the tissue surfaces, thus rendering them less anti-adhesive, and exposes extra-cellular matrix proteins that can aid repair and attachment (Hunziker & Rosenberg, 1996). Exposure of these proteins has also been proposed to contribute to enhancing tTG-catalysed bonding in response to shear stress, by increasing the accessibility of tTG to potential substrates in the cartilage matrix at the tissue surfaces (Jurgensen *et al*, 1997).

Collagenase digestion led to a significant decrease in the tensile bond strength of tTG at cartilage–cartilage interfaces, which may be due to it reducing the number of tTG-substrate residues available on the interface surfaces, by digesting away exposed

collagen fibres. This deleterious effect of the collagenase digestion could be countered by a subsequent GAG-digestion before tTG application, which then led to an enhancement of tTG bond strength as shown following a GAG-digestion alone. The GAG-digestion may therefore again have been revealing more tTG-substrate residues on the tissue surfaces, to counter those that had been removed following the collagenase digest.

GAG-digestions of longer than 15 minutes were not attempted, even though tTG-bond strength appeared to increase following GAG-digestion in a time dependent manner with respect to the duration of the digestion. This was because in a clinical situation, a longer digestion would not only prolong the adhesive application time for surgical users but has also been suggested to result in unnecessary and potentially harmful degradation of proteoglycans outside the cartilage defect area (Hunziker, 1992).

The treatment of tissue surfaces to aid bonding has also been proposed for fibrin sealant application. Exposing the collagen and fibronectin of tissues to the sealant fibrin might improve sealant bonding as fibrin bonds primarily to these substrates both covalently and non-covalently (Sierra, 1993).

In order to determine what factors might be aiding tTG-catalysed adhesion at cartilage-cartilage interfaces following GAG-digestion, various techniques were used to study the topography and biochemistry of cut, hyaline, costal-cartilage surfaces subjected to such digestions.

Cartilage surface topography was first examined by ESEM, SEM and confocal laser scanning microscopy. Using these techniques, no differences in structure could be observed between undigested tissue surfaces and those subjected to GAG-digestion prior to viewing. Whilst the published studies of cartilage surfaces have tended to concentrate on articular cartilage and the tissue surface presented at joints, the surface features of cut, hyaline cartilage observed in this study were consistent with the features noted in the literature (see IV-3.1, 3.2, 3.3).

A fundamental problem in analysing cartilage is examining the tissue in its native state. Cartilage is sensitive to many tissue preparation techniques because of its high water content, the removal of which can fundamentally alter the structure of the tissue.

This certainly occurred during CLSM analysis as the laser used for profiling the cartilage surfaces did lead to the tissues drying out rapidly. Environmental Scanning Electron Microscopy (ESEM) does allow wet samples to be viewed without harsh preparation techniques and the SEM analysis performed involved flash-freezing the cartilage in liquid nitrogen and viewing the tissue on a cryo-stage to try and preserve its hydrated state. But freezing the tissue meant that it still wasn't being observed in its natural state and the ESEM analysis did not allow the same area of sample to be viewed both before and after digestion of its surface GAG's. So any comparisons of the effect of the digestion were being made between different tissue samples. Also, the surface features observed from these studies, which matched those seen in conventional SEM studies of cartilage surfaces, have been suggested to be artifacts produced by dehydration during tissue preparation and analysis (Jurvelin *et al*, 1996).

Therefore the cut cartilage surfaces were also analysed using Atomic Force Microscopy (AFM), a technique that allowed the surfaces to be imaged under liquid and the same area of tissue to be examined before and after GAG-digestion.

Whilst AFM failed to reveal any fine structure details of the tissue that could be compared with published TEM and AFM analysis, it did show the surface topography of the cut, cartilage surfaces to be altered following digestion of its GAG constituent. Upon imaging the same region of tissue surface, before and after digestion with Chondroitinase AC, the microscopic topography of the cartilage surface was shown to become increasingly 'smoother' following the digestion. This effect may contribute to the increased adhesion shown by tTG at GAG-digested cartilage-cartilage interfaces, by allowing the two tissue surfaces to come into closer proximity. Thus providing tTG with more points of direct contact between the two surfaces, with which it may cross-link the interface together and promote adhesion. If, as suggested, TG protein concentration is a factor in tTG-catalysed bond strength as well as enzyme activity, then reducing the distance between the tissue interface may reduce the amount of auto-catalytic cross-linking required for tTG to span the interface and bond the two surfaces together. Leaving more enzyme available to increase the degree of cross-linking between the interface, increasing the amount of bonding between the tissue surfaces.

Digestion of a cartilage defect surface using CABC (1U/ml) for 4 minutes showed digestion of proteoglycans 1-2μm deep into the tissue, as demonstrated by EM

analysis (Hunziker, 1992). Referring to the maximum peak height shown in the AFM profiles, a CAC (1U/ml) digestion for 2 minutes showed a reduction in maximum peak height of approximately 0.6μm. If the reduction is attributable to the proteoglycan digestion, this rate corresponds well with that found in the previous study.

The availability of tTG-substrate residues on untreated and GAG-digested cartilage surfaces was estimated by looking at the degree of tTG-catalysed incorporation of fluorescent TG-substrate molecules into the tissue surface. An increased incorporation of fluorescein cadaverine (fluorescent TG-substrate polyamine) into a tissue surface by tTG indicates a greater number of potential TG-substrate glutamine residues being available thereupon. Fluorescein cadaverine has been used previously for the detection of amine acceptor protein substrates accessible to transglutaminase in rabbit articular chondrocytes (Lajemi *et al*, 1998) and fibroblasts (Verderio *et al*, 1998).

Using this method, GAG-digestion of cut cartilage surfaces was shown to increase the level of tTG-catalysed incorporation of fluorescein cadaverine into the tissue, in a time-dependent manner with respect to the duration of digestion. Indicating that GAG-digestion of a cartilage surface reveals more potential peptide-bound TG-substrate glutamine residues thereupon, allowing for the increased tTG-catalysed incorporation of fluorescent substrate.

The increased bond strength shown by tTG at GAG-digested cartilage-cartilage interfaces, compared to untreated ones, may therefore be due to:

1. The removal of negative charge.
2. Smoother surface topography (at a microscopic level) and decreased distance between interface surfaces.
3. An increased exposure of peptide-bound γ–glutamyl residues which allow for higher levels of tTG-catalysed bonding between the tissue surfaces.

The location of the exposed substrates is presumably on the collagenous constituents of the extra-cellular matrix and proteins such as fibronectin (as theorised by Jurgensen *et al*, 1997). Co-localisation studies, using fluorescein cadaverine and markers for cartilage-ECM constituents, may have revealed the whereabouts of the exposed residues and remains an area of possible future study.

The design of cartilage repair materials can now be tailored in order to maximise their potential for retention in defect sites by tTG-catalysed adhesion.

Increasing the number of tTG-substrate residues available on a biomaterial should allow for increased levels of tTG-catalysed bonding between the material and defect surface. More specifically, increasing the number of ε-lysine groups available on the material and digesting the cartilage surfaces intended for bonding to remove GAG's, should allow for increased levels of tTG-catalysed cross-linking at the tissue-biomaterial interface. Between the ε-lysine residues of the biomaterial and the γ-glutamyl residues exposed on the cartilage surface by the proteoglycan digestion. This should then lead to increased bond strength at the biomaterial-tissue interface, promoting adhesion and retention of the implant and helping to promote tissue repair.

The capacity of tTG to bond a biomaterial-cartilage interface was demonstrated with both the materials and cartilage surfaces being optimised for maximum tTG-cross-linking potential. GAG-digestion exposed peptide-bound γ-glutamyl residues on the cartilage surfaces whilst the biomaterials were synthesised to contain either multiple ε-lysine residues or TG-substrate ECM proteins containing both ε-lysine and γ-glutamyl residues (Jones *et al*, 1997; Fesus *et al*, 1986; Bowness *et al*, 1987; Aesclimann *et al*, 1993). These measures increased the numbers of potential TG cross-linking sites at the tissue-biomaterial interfaces, promoting tTG-catalysed bonding and adhesion.

The biomaterials used were poly-lactic acid, synthesised to include multiple ε-lysine groups, and poly(ε-caprolactone) synthesised to include the proteins collagen and fibronectin. Both of these base materials have previously been proposed for use in tissue repair. Poly- lactic acid is used in a wide range of medical applications such as biodegradable sutures, dressings, plates and pins (Chu *et al*, 1995). It has also had application as a matrix material for the reconstruction of articular defects in the rabbit (Von Schroeder *et al*, 1991), where it has also been used in conjunction with periochondirum cells, assisting repair by acting as a scaffold for cell migration and proliferation (Dounchis *et al*, 2000). Poly(ε-caprolactone) has been investigated for use as a long-term implant for drug release and as a co-polymer (with poly-lactic acid) for use in orthopaedic applications such as repair of bone defects (Rizzi *et al*, 2001). It has also been proposed as a matrix material for a bioresorbable composite for craniofacial reconstruction (Corden *et al*, 2000).

Thus tTG has been demonstrated to be capable of bonding repair materials that are already in use in orthopaedic repair.

Biomaterials are being increasingly used for cartilage repair, where they are implanted into defect sites to act as scaffolds for the remodelling of the defect site by implanted or host cells (see I-3.4.2.4). As with most cartilage repair technologies, the retention of repair material in defect sites has proved problematic, but these results demonstrate that tTG can be used to bind biomaterials to cartilage surfaces, help their retention and hopefully promote repair. Biomaterials may then be optimised both for the ability to act as a cell scaffold and for their retention in defect sites by tTG-catalysed cross-linking, through the inclusion of multiple tTG-substrate residues in the material structure.

GAG-digestion was one of several methods investigated to promote tTG-catalysed adhesion at cartilage-cartilage interfaces by altering the tissue surfaces to be bonded. In addition to this approach, the enzyme preparation itself was altered to try and aid bonding.

TG-substrate proteins and polyamines were added to the adhesive to act as structural bridges, that could be cross-linked by tTG to the cartilage surfaces and to each other, and help to span any gaps occurring along the tissue interface. This may then raise the bond strength of the tTG preparation. As well as using this approach at untreated cartilage-cartilage interfaces, lysine-rich TG-substrate proteins and polyamines were specifically added to preparations applied at GAG-digested interface, in order to provide increased numbers of available primary amine groups for cross-linking by tTG to the additional γ–glutamyl groups exposed on the tissue surfaces by the GAG-digestion.

The addition of TG-substrate proteins and polyamines to the tTG preparations was largely unsuccessful in raising bond strength. Polyamines, bovine albumin, casein, poly-l-lysine and fibronectin all had no positive effect on tTG bond strength at untreated cartilage-cartilage interfaces (and at GAG-digested interfaces in the case of putrescine, caesin and poly-l-lysine).

Collagen II did have a positive effect at untreated interfaces. This is encouraging as collagen II is the main structural component of cartilage and collagen is also used as a matrix material for cartilage repair (see I-3.4.2.4). Its inclusion in a tTG preparation

applied to a cartilage-cartilage interface may then both improve bonding and provide a cross-linked scaffold to fill any gaps occurring along the tissue interface. The scaffold would be composed of the same material as the surrounding tissue, which might facilitate a degree of intrinsic repair, or an appropriate cell type (e.g. chondrocytes) could also be included in the preparation to ensure new cartilage tissue is laid down along the interface.

A second protein, which was successful in raising tTG bond strength, was gelatin. This effect was deemed to be due to its being a TG-substrate rather than merely its ability to gel during testing at room temperature. Like collagen, gelatin has already been proposed for biomaterial applications. It forms the basis for biological adhesives, with the bonding strengths and biocompatibility of a gelatin / poly(l-glutamic acid) glue reported to exceed those of fibrin sealants (Otani *et al*, 1996) (see I-2.4). In tensile testing at butt-jointed cartilage-cartilage interfaces a Gelatin / Resorcinol / Formaldehyde adhesive produced a bond strength of 0.15 MPa, almost an order of magnitude greater than that shown by a commercial fibrin sealant at equivalent interfaces (0.049 MPa, Chivers & Wolowacz, 1997). Whilst gelatin can improve the bond strength of a tTG-based adhesive, the addition of tTG to a gelatin-based adhesive may succeed in improving the bonding of these preparations also.

Gelatin cross-linked by TG has been proposed as a system for enclosing bioactive molecules with no detrimental effect on their activity (Fuchsbauer *et al*, 1996). So the addition of gelatin to a tTG adhesive preparation may not only promote increased bonding when applied to a cartilage-cartilage interface, but also provide a delivery system for targeting bioactive molecules (e.g. growth factors) to cartilage defect sites. By enclosing the growth factor in a TG-cross-linked gelatin preparation, its retention at the site of application may be increased and its release rate perhaps controlled by altering the rate of breakdown of the gelatin / TG matrix.

This approach, of combining tTG with currently available or developing technologies, may be the key to producing an effective biological tissue adhesive or cartilage repair system. Tissue transglutaminase does not merely have to be used on its own to bond cartilage-cartilage interfaces. It can promote the attachment of a variety of repair materials to cartilage defects, may be used to alter and improve the properties of such materials, or conversely these materials themselves may promote tTG-

catalysed bonding. As was found for poly-lactic acid, poly-capro lactone, collagen and gelatin, tTG can be used to facilitate the bonding of cartilage to repair materials that are already in use for tissue engineering.

In terms of improving the properties of materials, the incorporation into biomaterials of peptides with high TG affinity or substrates that contain cross-linking sites for the enzyme may enhance cell attachment and promote biocompatibility of the material. As expression of tTG by human osteoblast and endothelial cells has been shown to promote biological recognition of poly-glycolic acid polymers, and positively correlate with cell spreading on such a surface (Verderio *et al*, 2000). The development of biomaterials, which can be colonised by cells that deposit their own ECM, and eventually degrade to leave newly formed tissue would be of benefit to tissue repair as a whole.

Whilst the inclusion of TG-substrate proteins in tTG preparations has mostly been unsuccessful in raising tTG bond strength, the theory may still be sound but the correct protein additive yet to be found. It may be that a specifically designed substrate molecule is required. TG cross-linkable polypeptides (of varying size containing Gln-Y-Lys where y is a spacer) have been proposed for use in a variety of homo- and co-polymer formulations that can be cross-linked by TG to add stability to biomaterials, form matrices and enable the biomaterial to adhere to tissue surfaces. These materials can be used to form adhesives, matrices, rigid prosthetics, wound repair formulations and controlled drug release compositions (Labroo & Busby, 1995).

As well as tTG, the capacity of other transglutaminases to bond cartilage-cartilage interfaces was also studied.

Neither microbial TG (Ajinomoto Co, Japan, from *Streptovercillium*) nor a commercial Factor XIII preparation were capable of bonding a cartilage-cartilage interface with the same strength as tTG. However a comparison between the different enzyme preparations is difficult as either the precise TG content or specific activity of the mTG and FXIII preparations is unknown. The lower bond strength of the mTG preparation, compared to the tTG solution, may have been due to differences in substrate specificity. This may also be the case for the FXIII preparation, which had a far lower specific activity than the tTG used.

An advantage to using the microbial TG as the basis for a tissue adhesive would be the possible mass production of the enzyme, already in use as a food-binding product (Ando *et al*, 1989; Yasuyuki *et al*, 1996). This may allow for pure, high concentration mTG preparations to be produced cheaply, which could lead to high bonding strength solutions, as the strength of protein gels cross-linked by mTG has been shown to increase with mTG protein content (Sakamoto *et al*, 1994). However in this study the bond strength of a mTG preparation did not increase with supposed increasing enzyme concentration. An adhesive based on a microbial enzyme may also elicit unwanted immune responses when used *in vivo*.

The work presented in this thesis has shown that tTG is capable of bonding cartilage-cartilage interfaces in response to tensile stress. The bond strengths achieved match, and can exceed, those of a commercially available biological adhesive under equivalent conditions. These strengths can be increased through the addition of certain tTG-substrate proteins to the enzyme preparation and also by various methods of pre-treating the cartilage surfaces to be bonded. Enhancing tTG bond strength further may be achieved through the design of specific TG-substrate proteins for inclusion in the preparation, an area for possible future research.

Further investigations into the tTG-catalysed bonding of other tissue types such as bone, skin and blood vessel, would also be of benefit as the need for an effective biological adhesive is not merely limited to the field of cartilage repair. It was mentioned earlier that the *in vitro* test system developed here allows for the direct comparison of different adhesive under equivalent conditions. The same may also be possible for different tissue types and the same (or varying) adhesive, once the test set-up is modified to accommodate the different tissue shapes and sizes.

One of the pre-treatments of cartilage surfaces that proved successful in raising tTG bond strength was the digestion of GAG's, which smoothed the topography of the cartilage surface and increased the number of exposed TG-substrate γ-glutamyl residues thereupon. This fact now facilitates the design of cartilage repair materials to allow for maximum tTG-catalysed bonding when implanted into cartilage defects. Cartilage repair techniques are increasingly likely to incorporate specially synthesised polymers and matrices, for implantation into defects along with a cell population capable of forming new tissue. The advantages of this form of therapy include:

1) minimal requirement for donor tissue,
2) the ability to specifically design the scaffold, and
3) the potential for arthroscopic implantation (Freed *et al*, 1994).

Therefore the development of an adhesive capable of bonding such biomaterials is of prime importance.

tTG has been shown to be capable of bonding two such optimised repair materials to cartilage surfaces and this potential means that future bonding studies would be worthwhile, using various current and proposed cartilage repair materials such as collagen fleeces, PLGA/PGA matrices, hyaluron matrices, fibrin matrices, tissue engineered cartilage constructs and perichondrial grafts. This will hopefully highlight the compatibility of the tTG adhesive preparation with current tissue repair materials.

Whilst tTG bond strength has been found to be greater than found for a commercial fibrin sealant, it is some way below those reported for the synthetic cyanoacrylates. Therefore its other proposed advantages over this class of adhesive such as increased biocompatibility and reduced toxicity should be investigated using *in vivo* studies. Such studies are also necessary for the continued evaluation of tTG bonding capacity.

In the final section of this thesis, chondrocyte viability at experimental wound edges within *in vitro* cultured articular cartilage explants was studied.

In studies of cartilage injury, a zone of cell death at the margins of lesion has been consistently noted, and it has been suggested that this may limit cartilage repair (Mankin, 1962; Hashimoto *et al*, 1998b; Tew *et al*, 2000). Areas of non-viable cartilage have been seen adjacent to defect drill-holes, that are 3-10 cell columns wide. These lacunae remain empty and so a small circumferential band of cartilage composing the wall of the defect remains dead and its extracellular matrix isn't replaced or repaired (Shapiro, 1993). The mechanism of cell death has previously been attributed to necrosis (Mankin, 1962) though recent studies have suggested the presence of apoptotic cells at the margins of experimental defects (Tew *et al*, 2000).

This band of dead tissue at wound margins is likely to compromise cartilage repair, which relies on integration between implanted/repair and native tissue, and it may also be a factor in affecting adhesive strength at bonded cartilage-cartilage interfaces. Adhesive strength at interfaces between bovine articular cartilage explants, maintained

in apposition in an *in vitro* culture system, was assessed as a measure of integrative cartilage repair and found to be dependent on viable cells (Reindel *et al*, 1995).

The *in vitro* model developed in this study used confocal laser scanning microscopy and a dual cell viability stain to assess cell viability and measured the distance the band of dead cells seen at the cartilage wound edges progressed into the tissue over time in culture.

Using the model, a band of cell death was indeed seen at experimental wound edges that progressed into the tissue over time. This loss of cell viability was more pronounced in serum-free culture conditions and also when the wound edge was removed from contact with the surrounding explant tissue. This suggests that contact with surrounding extracellular-matrix or chondrocytes helps to prevent cell death, possibly through a diffusible 'survival' signal emanating from the surrounding chondrocytes or matrix, which is lost when the wound edge is removed from contact with the tissue explant. Therefore if cartilage is to be harvested for the purpose of grafting, it may be beneficial to leave the graft in contact with its surrounding tissue until immediately prior to the procedure, in order that the viability of the cells at its margins be preserved.

This theory of contact promoting cell survival has been reported previously in similar studies of cell death around experimental wound edges created in articular cartilage explants (Thompson, 1999). Cell death was shown to decrease with increasing distance from the wound edge, being concentrated within around 80μm of the wound edge, and assay of DNA fragmentation was used to confirm apoptotic cell death. Cell viability was analysed using CLSM analysis of cores taken from cartilage explants, and replacing the core back into the explants and assaying cell death showed that there was a degree of suppression of cell death when the wound edges were in close proximity, or when the defects were filled with an agarose gel. It was suggested that a cell-cell signalling failure may be occurring at the wound edge that induces apoptosis and leads to the cell death observed, and that partial restoration of this signalling through close proximity of the wound edge to surrounding tissue helps reduce the loss of cell viability. These findings correlate with the responses of chondrocytes to experimental wounding in articular cartilage explants seen in this study.

A loss of extracellular-matrix survival signals has also been proposed as an inducer of apoptosis in articular chondrocytes (Hashimoto *et al*, 1998b). Observation of knee

cartilage from normal tissue and from osteoarthritis patients showed chondrocyte apoptosis to be linked to proteoglycan depletion and osteoarthritis grade.

The loss of chondrocyte viability seen at experimental wound edges in this study was significantly increased when the wound edge was subjected to GAG-digestion using CAC (1U/ml for 15 minutes). This occurred when the digestion was performed either post-wounding but prior to culture, or following both wounding and culture for 1 or 7 days. The fact that cell death was increased when the digestion was performed after culture and immediately prior to the assessment of cell viability, shows the effect on cell viability to be rapid.

So whilst GAG-digestion of cartilage surfaces may promote the attachment of repair materials (Hunziker & Rosenberg, 1996) and aid tTG-catalysed bonding of cartilage-cartilage interfaces in response to both shear and tensile stress (see III-3.5.4, Jurgensen *et al*, 1997) it decreases the level of chondrocyte viability at the cartilage surface. Meaning that any material bonded to the tissue surface, whether cartilage, soft tissue or biomaterial etc, will be binding to an area of largely dead tissue, greater than would be found at an untreated cartilage surface. GAG-digestion may therefore actually be detrimental to long-term cartilage repair and its use might be compromising existing repair technologies.

In terms of the longer term effects of GAG-digestion, CABC digestion of cartilage defect surfaces *in vivo* has been shown to only have a transient effect in increasing coverage of the defect by repair cells (Hunziker & Kapfinger, 1998). Six months following treatment, coverage of treated and untreated lesions were similar and not significantly different from that achieved in untreated defects at one month. So if GAG-digestion was removed from cartilage repair strategies in order to preserve cell viability at defect surfaces, it may not be detrimental to the repair in terms of coverage of the defect with repair cells.

The reason why GAG-digestion promotes a loss of cell viability at experimental cartilage wound edges may be due to the loss of cell/matrix interactions. If chondrocyte viability is indeed influenced by a 'survival signal' from surrounding cells and/or matrix, then the digestion may have disrupted matrix further into the tissue and enhanced the loss of the signal as felt by chondrocytes at the wound edge. Degradation of proteoglycans has previously been linked to chondrocyte apoptosis in human knee cartilage (Hashimoto *et al*, 1998b). Where the degradation of the ECM

was suggested to either result in the loss of a survival signal for the cells (as suggested in this study) or increase the susceptibility of the cells to undergo apoptosis in response to other stimuli.

Disruption of the extra-cellular matrix has also been shown to induce apoptosis in mammary epithelial cells both in culture and *in vivo* (Boudreau *et al*, 1995). Where signals derived from basement membrane ECM were thought not only to maintain the cells but also suppress apoptosis. This apoptotic cell-death induced by disruption of cell-matrix interactions has been termed 'anoikis' (Frisch & Francis, 1994). The Jun N-terminal kinase (JNK) pathway is activated in and promotes anoikis and activation of its initiating kinase (MEKK-1) requires caspase-mediated cleavage (Cardone *et al*, 1997). Thus caspase activity may be one mechanism by which anoikis is regulated.

The mechanism by which chondrocytes were dying at the experimental wound edges created in this study was not extensively studied.

The cell death at cartilage wound edges has been attributed to both necrosis and apoptosis (Mankin *et al*, 1962; Tew *et al*, 2000). In a study of cell death at experimental cartilage wound edges *in vitro*, TUNEL labelling was shown to increase in the first 200μm of tissue from the wound edge (Tew *et al*, 2000). However TUNEL is an unreliable method for defining apoptotic cells. Cells directly adjacent to the wound edge (likely to have be mechanically compromised) showed no apoptotic structural characteristics, but were still TUNEL positive. It may be that the chondrocyte cell death is occurring by a combination of necrosis and apoptosis. TUNEL positive cells progressed into the tissue over culture time as was found for the band of cell death measured in our *in vitro* model. Chondrocyte apoptosis has also been observed in response to mechanical compression of bovine articular cartilage explants (Loening *et al*, 2000). In agreement with the previous study by Tew *et al* (2000), numerous cells at the cut edges of both loaded and unloaded tissue stained positive for apoptosis by TUNEL staining. But loaded tissue also showed an increase in apoptotic nuclei and the appearance of nuclear blebbing in the central region of the tissue away from the cut edges, where the cutting should have had no effect.

Caspases are a family of proteases that cleave a specific subset of cellular polypeptides, whose cleavage is one of the intracellular changes that are characteristic of apoptotic death (Earnshaw *et* al, 1999). In the present study, the presence of a

general caspase inhibitor did not reduce the amount of cell death seen at experimental cartilage wound edges. A response also observed in previous studies (Tew *et al*, 2000).

The inability of a caspase inhibitor to reduce cell death at experimental wound edges may suggest that caspase-mediated apoptosis is not the only form of cell death occurring at the experimental wound edges. Equally, chondrocytes at the wound edge may not have been exposed to sufficient levels of the inhibitor and further studies may show caspase inhibition to indeed be beneficial in reducing cell death at experimental cartilage wound edges. The selective inhibition of caspases 3 and 7 has been shown to inhibit campothecin-induced apoptosis in human chondrocytes in a manner that maintains cell functionality (Lee *et al*, 2000), suggesting that caspase inhibition may be an effective therapeutic treatment for prevention of apoptotic cell death in chondrocytes. If proportions of the cells at cartilage wound edges are dying by a process of apoptosis, then its inhibition may reduce this loss of cell viability that would then have beneficial implications for cartilage repair.

Use of a tTG preparation to bind the cartilage-cartilage interface formed between experimental wound edges in articular cartilage explants had no detrimental effect on chondrocyte viability. Indicating that any possible disruption of the cartilage extracellular matrix caused by the cross-linking action of tTG does not lead to an increased loss of chondrocyte viability. This is encouraging for the application of tTG-based adhesives at cartilage-interfaces during repair *in vivo*.

The tTG-catalysed bonding of experimental wound edges was thought to have potential in reducing the loss of chondrocyte viability that occurs, by its cross-linking action reducing the gap at the interface between the wound edge and the rest of the tissue explant, producing a sealing effect. Since contact with surrounding explant tissue was shown to reduce cell death at experimental wound edges, tTG-cross-linking might have reduced chondrocyte cell death further by keeping the wound edge in more direct contact with the surrounding tissue, but this was not the case. Whether tTG-cross linking would be effective in reducing cell death at cartilage wound edges if applied in greater amounts or in conjunction with a substrate protein such as collagen II, remains a possible area of study. As is bonding the wound edge interface directly with an activated tTG adhesive (in a method similar to that used during *in vitro*

measurement of tTG-bond strength), rather than coating one side of the interface with tTG protein and then activating the enzyme using calcium present in culture media.

It would be interesting to use this model for direct a comparison into how the application of other tissue adhesives affect cell viability at bonded cartilage-cartilage interfaces. This may give an indication into how the adhesives would affect cell viability and cartilage repair *in vivo*. Preliminary experiments of this nature, using Beriplast® fibrin sealant, were began but not extensively investigated (data not presented in this thesis). The effect of cyanoacrylate tissue adhesives on cartilage graft viability has been studied using human tissue and four different cyanoacrylate adhesives (Quatela *et al*, 1993). A significant decrease was seen in the viability of tissue bonded with three of the adhesives, whilst Histoacryl-bonded specimens showed no difference compared to controls. As was previously suggested for the measurement of bond strength, the *in vitro* model developed for assessing chondrocyte viability at experimental wound edges may be used to provide direct comparison of tissue bonding methods.

Using the *in vitro* model presented, the level of cell death seen at cartilage wound edges was shown to increase following treatment of the tissue to remove GAG's. It may therefore be beneficial to also use the model to investigate the effects of other cartilage repair techniques on chondrocyte viability at the experimental wound edge. This may include studying the effects of implants (tissues and biomaterials) and treatment methods such as debridement, as well as adhesives. A previous study, also using a double-staining CLSM protocol, investigated chondrocyte viability within poly-lactic acid polymer matrices (Chu *et al*, 1995) and it was shown that chondrocytes remain viable when cultured in such a scaffold. The implantation of such a material into the wound defect created in our model would highlight what effect the material has on cell viability at defect margins within the recipient tissue.

A concerted study into the mechanism of cell death occurring at cartilage wound edges is also possible using the *in vitro* method developed for assessing cell viability. This may facilitate the development of therapeutic treatments that prevent the loss of chondrocyte viability and promote tissue repair. Such as the use of sharper surgical

instruments during cartilage procedures which is suggested to reduce matrix disruption and subsequent cell death (Tew *et al*, 2000).

One such therapy may be the application of inhibitors of nitric oxide synthesis to cartilage wound edges.

Chondrocytes produce nitric oxide (NO) and undergo apoptosis in response to exogenous NO . Following experimentally induced osteoarthritis (OA) in rabbits knees, an increased prevalence of apoptotic cells was observed in the cartilage that correlated with the grade of OA and levels of nitrite production (Hashimoto *et al*, 1998). NO production is also associated with apoptosis in meniscal cells during experimental osteoarthritis and nitric oxide donors can induce chondrocyte apoptosis *in vitro* (Hashimoto *et al*, 1999a; Blanco *et al*, 1995). Whilst the role of endogenously produced NO in chondrocyte apoptosis remains unclear, chondrocyte death is thought to play an important part in the pathogenesis of OA. Since NO production may contribute to chondrocyte apoptosis, inhibitors of NO synthesis and chondrocyte apoptosis may be of therapeutic value following both cartilage injury and in joint degenerative disorders such as OA. Indeed, the reduced progression of experimental OA in a dog model was achieved by administration of the selective inhibitor of inducible nitric oxide synthase (Pelletier *et al*, 1998).

The level of cell death at experimental cartilage wound edges was also shown to be unaffected by culturing the tissue at low oxygen levels (5% O_2 compared to controls at 20% O_2).

The long-term survival of grafted tissue depends on the continued viability of its cells (Newman, 1998). If cartilage tissue is to be grown or stored in culture before implantation into defects, then the establishment of optimum culture conditions for preserving graft viability and tissue functionality will help maximise the potential for tissue repair. The *in vitro* model developed during this work, for evaluating cell death at experimental cartilage wound edges, may be used for determining the viability of cartilage tissue maintained in culture in response to varying conditions. Thus helping to establish optimum culture conditions for the preservation of graft tissue.

Since cartilage is an avascular tissue with low oxygen tension *in vivo*, reduced oxygen conditions have been investigated as a means of preserving chondrocyte phenotype and promoting proliferation during *in vitro* culture. Primary human chondrocytes and a mouse chondrocytic cell line have both been shown to maintain or

elevate their proliferation rates when cultured in monolayers at low oxygen levels (35 mm/Hg compared to the 120mm/Hg occurring in standard CO_2 incubators) (Cooling *et al*, 2000). For chondrocytes cultured encapsulated on alginate beads, reduced oxygen (5% vs. 20%) gave no difference in cell number over the 4-week culture period but did promote collagen II expression and led to higher levels of GAG's in the surrounding matrix (Murphy & Sambanis, 2000).

Whilst reduced oxygen conditions did not improve chondrocyte viability levels at experimental wound edges within articular cartilage explants, it may still have helped maintain the quality of the tissue in terms of promoting expression of matrix proteins and maintaining chondrocyte phenotype.

A proliferative response has been observed in chondrocytes behind the zone of cell death at experimental cartilage wound edges (Tew *et al*, 2000) and culturing cartilage tissue *in vitro* at low oxygen levels may help promote this proliferative response, aiding intrinsic repair of the tissue margins.

The distance that the bands of dead cell progressed into the tissue varied with culture conditions (as discussed). On average, after 7 days culture the width of the band of dead cells at control tissue punch wound edges was around 30-40μm (see VI-III). The greatest width of cell death measured was 110μm, seen at a wound edge subjected to GAG-digestion after both wounding and culture for 7 days. The studies of Thompson (1999) reported cell death to be concentrated to approximately 80μm from experimental wound edges, although the time elapsed between wounding and measurement is unknown.

In summary, the capacity of tTG to act as a cartilage-bonding agent has been investigated using a novel *in vitro* test system for measuring bond strength at cartilage-cartilage interfaces. It has been found to have promise in this regard, being able to bond such interfaces with equivalent and exceeding strengths to those shown by a commercially available biological tissue sealant over a range of incubation times and humidities. The bond strength of tTG can be facilitated by methods of pre-treating the tissue surfaces and by alteration in the composition of the adhesive. With the digestion of GAG's, scoring of the tissue surface, drying of the tissue surface, inclusion of gelatin and inclusion of collagen (II) all leading to increased bond

strength of tTG at cartilage-cartilage interfaces. As well as cartilage-cartilage interfaces, tTG has been shown to be capable of bonding interfaces between cartilage and synthetic polymers, which have had application as biomaterials for tissue repair. The novel test method developed allows for the direct comparison of the bond strengths of both tTG and other adhesives at a variety of tissue and material interfaces. This comparison is currently lacking as adhesive investigations often have test systems individual to each investigator.

A further method was developed for measuring cell viability levels at experimental wound edges created within articular cartilage explants *in vitro*.

Investigations using this system showed that application of tTG to a cartilage wound edge interface did not have a detrimental effect on cell viability at the margins of the recipient tissue. However, digestion of GAG's from the wound edge surface did lead to an increased loss of cell viability.

As before, this system allows for direct comparison between treatments proposed for cartilage repair and measurement of their effects on cell viability at wound edges *in vitro*. This will give an indication as to the tissue response *in vivo*. Comparison of the treatments investigated in this study show that compared to the level of cell death seen at control wound edges, both the presence of serum and contact with surrounding explant tissue reduce the loss of chondrocyte viability seen at experimental wound edges, to around 50% of control levels. Whereas GAG digestion led to widths of cell death at experimental wound edges twice those seen in the controls.

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