THE PRODUCTION OF HUMAN MATERIAL FOR SKIN REPLACEMENT

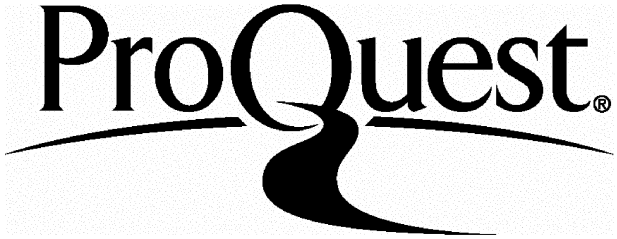
A thesis submitted to the University of London as part of the requirements for the degree of Doctor of Philosophy

BY

Sarah Anne Underwood

1999

The Advanced Centre for Biochemical Engineering
Department of Biochemical Engineering
UNIVERSITY COLLEGE LONDON
Torrington Place
LONDON WC1E 7JE



ABSTRACT

This thesis describes the research to identify a process for the production of cell guidance scaffolds with potential use as human skin replacements.

Fibronectin, a plasma protein with well-documented cell adhesion properties, can be extracted from the cryoprecipitate collected from human plasma donations. Purified solutions of fibronectin have been used to make mats with approximate dimensions 1 x 2 cm and consisting of orientated, protein fibrils with diameter 5-10 μm. These mats have demonstrated excellent *in vitro* and *in vivo* cell guidance properties. The aim of this research was to produce scaffolds with the same cell guidance / adhesion properties as the mats combined with a simple manufacturing method, little material wastage, reproducibility and potential for large scale production.

Polyethylene glycol fractionation was used to produce fibronectin enriched precipitate from cryoprecipitate, with fibronectin and fibrinogen in a 65 : 35 percentage ratio. The precipitation of fibronectin and fibrinogen with a reduction in pH was examined and it was found that fibronectin / fibrinogen cables could be drawn up from a solution when protein was precipitated with 0.1 M citric acid, final pH 4.0-4.5. These cables, average diameter 200 μm, were found to consist of numerous micron-diameter fibrils giving the cable ultrastructural orientation. The cables were found to be hygroscopic and could be stabilised against solubilisation by severe dehydration. Cables which were twisted as they were drawn upwards from solution had an average ultimate tensile strength of 61 N/mm^2, but were brittle. Although these cables could be made easily and were found to have good cell adhesion properties, drawing upwards from solution is not a technique applicable to large scale manufacture.

Development of wet-spun fibres using traditional textile manufacturing techniques required that both the solution viscosity and protein concentration were increased. Urea was used as a solvent to increase protein concentration and sodium alginate was used to increase the viscosity of the solution and alter its flow behaviour. A variety of coagulation baths were tested in order to find the optimal pH and salt concentrations for fibre formation. Fibres spun on a pilot scale wet-spinning rig, diameter 500 μm, were

found to have properties which varied with their composition and post spinning treatments. The fibres had a lower tensile strength, 28 N/mm^2, than the drawn, twisted cables but had a higher elongation at break, 52.4 % compared to 3.5 %, making them easier to handle. Wet spinning has been shown to be a suitable and flexible process for the production of fibronectin / fibrinogen fibres.

The production of fibres is considered with respect to process design, virus inactivation and GMP considerations. A test of the fibronectin material's ability to support the attachment and alignment of human dermal fibroblasts is also described.

ACKNOWLEDGEMENTS

The interdisciplinary nature of tissue engineering is reflected by the broad spectrum of people I have had the privilege of working alongside during this project. The enthusiasm shown by all has been the key to this stimulating and enjoyable challenge.

My thanks to:

Prof. Peter Dunnill (UCLACBE), Dr. Robert Brown (UCL, Tissue Repair Unit) and Dr. Alex MacLeod (Protein Fractionation Centre, Edinburgh) for their supervision, encouragement and thought-provoking discussions.

Dr. Andrew Afoke at the Division of Design and Mechanical Engineering, University of Westminster for his helpful advice and assistance with the tensile testing.

Drs George East and Vic Rogers at the Department of Textile Industries, University of Leeds for their advice on wet spinning and an enjoyable and productive fortnight using their pilot scale equipment.

The Development department at the Protein Fractionation Centre, Edinburgh for their assistance in preparing the fibronectin precipitate and several enjoyable weeks in Edinburgh

Kirsty Smith at the Tissue Repair Unit for assistance with scanning electron microscopy and Stuart Pope at UCL for his photographic skills.

I would also like to extend my appreciation to:

My tea-drinking and cake-eating companions in Room 115 for 3 years of varied and amusing discussion. One day the self-filling cake-tin will become reality. The Tissue Repair Unit Team for adopting me into their department. Members of the University of London Orienteering Club for numerous opportunities to get away from it all, usually lost in a forest. Maggie, Ali, Lilla and Helen for being great fun. William for his patience, support and 'outrageous' humour and my family for their support, encouragement and excellent humour throughout.

This work was supported by the BBSRC and a CASE studentship from the Protein Fractionation Centre, Scottish National Blood Transfusion Service, Edinburgh.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

h	height of liquid in rheometer	m
K	fluid consistency index (power law equation)	mPa s^n
L	capillary length	m
M	torque	mNm
n	fluid behaviour index	-
Q	flowrate	m^3s^{-1}
R	regain	%
R^2	linear regression coefficient	-
R_b	radius of bob	cm
R_c	radius of cup	cm
r	bob / spinneret radius	m
T	residence time in spinneret	s
w_d	dry weight of material	g
w_w	weight of water in material	g
γ	shear rate	s^{-1}
γ_w	capillary wall shear rate	s^{-1}
γ_{av}	mass average shear rate	s^{-1}
μ	viscosity	mPa s
μ_a	apparent viscosity	mPa s
τ	shear stress	mPa

1.0. Introduction

Organisation of the thesis

Since the spectrum of material covered in this thesis is quite wide, the introduction discusses the major points of a number of diverse issues. A description of the materials and methods used are given in chapter 2, with more detailed elements being dealt with in the appendices (chapter 10). There are 5 chapters containing results and in each chapter the results are discussed with reference to the appropriate literature. The first of these chapters, chapter 3, describes the development of assays and discusses the protein solution available for this research along with an investigation into fibronectin mat-making. Chapter 4 identifies an alternative method for producing fibronectin materials and looks at some of the new material's physical properties. The materials discussed in results chapters 3 and 4 have been made by small scale manufacturing techniques and so chapter 5 identifies key features required for the scale-up of a fibronectin material manufacturing process whilst chapter 6 gives the results of a pilot scale study. Chapter 7 gives results of an *in vitro* test of the cell adhesion and orientating properties of the materials produced by the small scale method of fibronectin material manufacture, described in chapter 4. A scaleable process for the production of fibronectin contact guidance materials is suggested in chapter 8 and Good Manufacturing Practice issues associated with the chosen process are discussed together with virus inactivation issues, critical when dealing with blood products. An overall discussion is presented in chapter 9 along with some conclusions about the work discussed in this thesis and some proposals for future study in this very exciting field. References for the entire thesis have been grouped together after the appendices.

1.1. Tissue engineering

The use of the phrase 'tissue engineering', certainly in the USA, originated at a National Science Foundation meeting in 1987. This heralded the recognition of tissue engineering as an emerging technology and it was defined as follows:

> 'Tissue engineering' is the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure/function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions.'

(Nerem, 1992)

Tissue engineering has now developed into a highly interdisciplinary field, encompassing aspects of engineering, molecular and cell biology and materials science leading to the formation and regeneration of tissues and organs. In 1994, the total revenues for tissue engineering, bioartificial organs and cell therapies were only $13 million but predictions for the world market in the year 2000 are for growth to more than $2.7 billion (Miller and Peshwa, 1996). This figure is higher than the predictions described by Johnson Langer (1996). She noted that the annual growth rate to the year 2000 has been forecast as 5-10 % and that estimates of the total market for artificial organs and tissues are expected to exceed $1 billion. In 1996 there were 30 companies, in the US, involved in tissue engineering, employing more than 1000 employees in total and having annual expenditures greater than $250 million (Johnson Langer, 1996).

An estimate of the cost of treatment, support and loss of productivity for people suffering tissue loss or organ failure in the US may exceed $400 billion annually (Hubbell and Langer, 1995). The US chronic wound market is believed to be worth $7 billion annually whilst that for severe thermal burns is estimated at $30 million and partial thickness burns at $300 million (1997 figures). Some examples of the number of surgical procedures carried out and therefore potential market sizes for tissue engineered products are given in Table 1.1.

Indication	Procedures or patients per year
Skin	
Burns	2 150 000 (150 000 hospitalized, 10 000 die)
Pressure sores	1 500 000
Venous stasis ulcers	500 000
Diabetic ulcers	600 000
Spinal cord and nerves	40 000
Tendon repair	33 000
Ligament repair	90 000
Blood vessels	
Heart	754 000
Large and small vessels	606 000

Table 1.1. *Incidence of surgical procedures carried out in the US related to certain deficiencies, which may eventually be treatable by tissue engineered products. Taken from Langer and Vacanti (1993).*

Langer and Vacanti (1993) identified three approaches to tissue engineering:

1) Isolated cells or cell substitutes where only the cells that supply the required function are introduced.

2) Tissue-inducing substances, including delivery of signal molecules, such as growth factors.

3) Cells incorporated into the body attached to matrices or encapsulated.

Treatment of the conditions (tissue or organ loss) mentioned in Table 1.1. can be by either reconstructive or transplantation surgery. Reconstruction requires the use of prosthetic materials or tissue transplants whilst transplantation requires donation of tissues or organs (Langer *et al.*, 1995). Transplantation is limited by donor shortages in all areas, thus the development of tissue engineered products becomes critical to meet this shortfall.

As this thesis concentrates on the development of a scaffold for skin replacement therapy, further discussion of tissue engineering will be narrowed to include only scaffold formation and tissue replacement with these. In general, a three-dimensional,

biodegradable polymer scaffold is seeded with the required cell type *in vitro* and then implanted into the body. The implanted scaffold acts as a template for the development of new extracellular matrix. Cells such as chondrocytes, tenocytes, hepatocytes, enterocytes, urothelial cells, osteocytes or dermal fibroblasts have been isolated and allowed to grow on scaffolds in attempts to mimic cartilage, tendon, liver, intestine, urothelium, bone and skin respectively, (Langer and Vacanti, 1993, Langer *et al.*, 1995) whilst scaffold materials have included a wide range of biomaterials. Synthetic polymers, including poly (L-) glycolic acid, polyvinyl alcohol and poly L-lactic acid have been used in the form of fibres, mesh or sponges (Langer *et al.*, 1995).

Selection of scaffold materials is discussed by Bell (1995) and Peters and Mooney (1997). Consideration should be taken, when choosing the appropriate material to support cell growth, as to whether a biodegradable or permanent scaffold is required. The toxicity of the scaffold material, whether mechanical strength is required or resistance to compressive forces, the interaction of the material with cells, how it can be formed e.g.: films, sponges, fibres and whether it is of low immunogenicity and thrombogenicity must also be considered along with cost and availability. The porosity of the material is critical to the transport of oxygen to cells and the migration of cells within the material, Table 1.2. The main decision is often between a natural material, not always available in large quantities but with proven cell adhesion properties and low immunoreactivity, versus a synthetic material, often cheaper, reproducible and relatively abundant but with less well documented cell adhesion properties and which may react adversely with the patient's immune system. There are advantages to both types of scaffold and ongoing research is attempting to combine these. Polymers combining both sets of properties are discussed in section 1.6.

Substance	diameter < 2 nm	Pore diameter 2 nm <diameter< 10 μm	diameter > 10 μm
	Microporous e.g.: microcapsulation of cells	Mesoporous e.g.: polyglycolic or polylactic acid matrices	Macroporous e.g.: polyglycolic or polylactic acid matrices
Oxygen	4 seconds	4 seconds	4 seconds
Human albumin	--------	170 seconds	170 seconds
Endothelial cell	--------	-------	2.5 hours

Table 1.2. *Time for substances to travel 100 microns through matrices with various pore sizes. Taken from Peters and Mooney (1997). The porosity of the scaffold and the final implant must allow both nutrient delivery and waste removal, exclusion of unwanted cells or materials, protein transport and cell migration.*

1.1.1. Skin replacement technologies

Skin was the first tissue engineered product to reach the commercial market and has been extensively researched. Tissue engineered skin replacement technologies are broadly aimed at two separate markets, burns victims and patients with ulcers. In the first case a patient with serious burns may require a large quantity of new material, more than can be supplied by the traditional route of autografting. For serious burns, covering >60 % total body surface area, the cost of a new technique may be immaterial compared to patient survival, reduction of wound contraction and scarring and maintenance of joint mobility. Patients suffering from ulceration are seeking complete wound healing, however as these problems are often chronic, with some underlying complication, cost of treatment and the cause of the initial problem should not be overlooked. The state of the wound bed onto which the skin replacement is received will differ in both cases.

The structure of healthy skin will be discussed briefly, followed by the important characteristics which should be mimicked in the production of a tissue replacement. The development of a number of skin replacements will be highlighted with an outline of those products currently on the market.

1.1.1.1. Skin structure

Skin has a bilayer structure with the upper, 0.1 mm thick, cellular epidermal layer attached to the lower, relatively acellular dermis. The epidermal layer cell population consists of 95 % keratinocytes, which act as a barrier against water loss and infection, as well as a small number of Langerhans cells and melanocytes. It is a predominantly avascular layer and contains little extracellular matrix, apart from a layer of collagen between the epidermis and dermis.

The dermis is much thicker than the overlying epidermis and lies above a fatty layer. The few cells present in the dermis are fibroblasts, which secrete the extracellular matrix, a proteinaceous structural layer, containing the proteins collagen and elastin as well as glycosaminoglycans. Blood vessels, nerve endings and sweat glands are all found in the dermal layer (Wood and Raxworthy, 1994).

Amongst the major functions of skin are protection against physical, chemical and biological injury, prevention of water loss, provision of receptors for pain, pressure, touch and temperature, protection against u.v. light, conversion of precursors to Vitamin D, heat regulation, removal of excretory products via sweat and absorption of lipid soluble substances (Ross *et al.*, 1989).

1.1.1.2. Requirements of a skin replacement and their use in burn injury

Characteristics required of replacement skin products are fast adherence to the wound bed, prevention of evaporative loss and avoidance of a build up of fluid from the wound. The graft needs to be flexible, durable and tear-resistant as well as act as a barrier to micro-organisms. It should not invoke an inflammatory response but be sterilizable and non-toxic and be easily removable, if acting as a temporary dressing, as well as cost effective (Hansbrough, 1984).

Different types of skin replacements are required for different injuries. Research has centred around burn wounds due to the potential mortality associated with such cases despite a large, and ever expanding, chronic wound population. Burns are the most frequent cause of large skin loss and are characterized according to the depth of the injury.

First-degree burns - injury only to the epidermis and do not usually require grafting.
Second-degree burns - regarded as superficial or deep depending on the degree of destruction of the dermis.
Third-degree burns - destruction of epidermis and dermis and can only be re-epitheliazed from the edges of the wound. However, keratinocytes migrate very slowly at wound margins, approximately 0.5mm per day, (Wood and Raxworthy, 1994) therefore skin replacement is essential to prevent fluid loss.

Burns are also classified by the amount of the body's surface area that they cover. Second or third degree burns which cover 20-30 % of the body's surface area are closed with split thickness autografts taken from donor sites on the body. When a greater area of the body is affected it is difficult to find enough autologous donor sites to cover the wound and skin replacement therapy would be required. It is most critical to maintain the barrier function of the epidermis after injury although the replacement of dermal architecture plays an important role in preventing wound contraction and increasing the durability of the repair. Thicker grafts show less wound contraction than a thin layer of cells (Gallico and O'Connor, 1995).

1.1.1.3. Development of skin replacements and those currently available commercially.

Products which have been developed to assist healing of skin wounds can be divided into three categories. Those which replace only the epidermal, keratinocyte layer are described as 'epidermal equivalents' whilst those replacing only the lower dermal layer are called 'dermal equivalents'. If both layers are replaced then the product is described as a 'skin substitute'.

In the following sections both biosynthetic (no living cells) and biologic (containing living cells) products will be described.

Epidermal equivalents

Cellular products can either be derived from the patient themselves and are then described as autologous or derived from one donor and transplanted into a different recipient and are then termed allogenic. Conventional skin grafting involves formation of a skin mesh or split skin graft from tissue removed from a donor site. This expands

the area of replacement tissue approximately 4 fold. In the early 1960s advancing tissue culture techniques by Rheinwald and Green resulted in enhanced culture of keratinocytes *in vitro*. Using these techniques a small skin biopsy, 2 cm^2 , can be used to produce a keratinocyte sheet to cover the entire adult body surface area in less than 1 month (Navsaria *et al.*, 1995). Whilst autologous cells are being expanded by this method, grafts from cadaver skin may be used to maintain a complete cellular covering. Unfortunately this procedure may pose viral transmission and immunoreactivity risks but may assist re-epithelialization by secreting growth factors.

Limitations to the production of sheets of replacement cells include their mechanical fragility, susceptibility to microbial contamination, the increased time whilst these autografts are prepared, poor attachment to the underlying fat layers where there is no dermal replacement, as well as wound contraction, easy blistering of the epidermis and high cost. Cost estimates for keratinocyte sheets range from $1 000 to $13 000 per percentage of body surface area covered. Combining the epidermal equivalent with a dermal equivalent has been shown to eliminate the blistering of the newly formed epidermis, however this can double the cost (Boyce, 1996).

A commercially available epidermal cell autograft, 'Epicel', originally developed by BioSurface Technology Inc. (Cambridge, MA) and now by Genzyme Tissue Repair (Cambridge, MA) has been marketed in the USA since 1988 for treatment of severe burns. Patients own cells are removed by biopsy, expanded in culture over 3-4 weeks, attached to a vaseline backing and returned to the clinician for grafting. Total sales for 1993 reached $5.4 million and increased to $6.3 million in 1994. The selling price of 'Epicel' in 1995 was $15 000 per square foot (Glaser, 1995) and estimates of cost to treat one patient in 1993 were made as $62 000 (Szoka, 1993) whilst the cost of a full-thickness autograft was estimated as $6 000. The same company are also marketing a non-autologous product for 'off the shelf' treatment of burns and ulcers called 'Acticel', whilst FIDIA Advanced Biopolymers (Padova, Italy) are developing a keratinocyte delivery system, 'Laserskin', using a hyaluronic acid derived backing.

Dermal equivalents

A number of dermal equivalents have been developed, including Integra™, Dermagraft-TC, Alloderm and Biobrane. Details of their structure and usage are given in Table 1.3. All these provide a matrix for dermal regeneration and should be covered with an autograft. Dermal equivalents reduce the scarring and wound contraction associated with application of autografts to a full-thickness wound with no dermal component. Unfortunately all these products have some potential disadvantages. Integra™'s collagen component is from a bovine source whilst Alloderm originates from cadavers, both therefore possessing a pathogen transmission risk. The silicone derived layer covering the Biobrane or Dermagraft dressings must be removed before autografting occurs and it is important that the wound bed is thoroughly healed before this is done.

Collagen has a very low immunoreactivity and is therefore popular for use in tissue replacements. However problems with source, increased collagenase activities in wound beds and microbial infection have encouraged the use of synthetic polymers, such as poly glycolic acid, used in Dermagraft-TC and nylon in Biobrane.

Other products are still being developed by these companies for alternative functions, e.g.: Dermagraft for treatment of diabetic foot ulcers, which will be based on the same technology as Dermagraft-TC but it will be a permanent treatment. LifeCell also produce Xenoderm, a porcine based dermal equivalent for burns treatment, whilst both Advanced Tissue Sciences and Organogenesis produce materials for cosmetic testing.

Skin substitutes

Apligraf®, produced by Organogenesis, contains both allogenic dermal and epidermal layers and acts as an 'off-the-shelf' skin substitute. There are no Langerhans cells in the keratinocyte layer which is believed to make the product immunologically inert whilst the keratinocytes present may deliver growth factors to the wound, improving healing. Clinical trials, treating venous ulcer patients, showed promising results when compared to standard compression therapy. Wound contraction was measured at 10-15 %, which is slightly higher than full-thickness grafts, but there was no toxicity or rejection and the materials were easy to handle.

A number of other groups are researching methods of manufacturing skin repair products largely based on collagen (Lillie *et al.*, 1980; Doillon and Silver, 1986; Doillon *et al.*, 1987, 1994; Matsuda *et al.*, 1993; Maruguchi *et al.*, 1994; Berthod *et al.*, 1996) or gelatin (Yoshizato and Yoshikawa, 1994).

1.1.2. Future developments within tissue engineering

The first tissue engineered products to receive FDA approval were artificial skins but there are numerous other products under development, some using similar scaffold based technologies, such as conduits for nerve regeneration in the peripheral and central nervous system or scaffolds for the growth of hepatocytes in the production of artificial liver tissue. Scaffolds for chondrocytes, for use in cartilage replacement, are well advanced (Freed *et al.*, 1994) whilst tubes have been made from similar scaffolding materials for the development of blood vessels, intestinal tissue, tracheas and ureters (Mooney *et al* ., 1994, 1996). Replacements for bone, muscle and corneal tissue are also being investigated, (Langer and Vacanti, 1993) as well as possibilities for implanting insulin-secreting cells enclosed in semi-permeable membranes to mimic the function of the pancreas or using encapsulated cells to secrete dopamine in the brain in cases of Parkinson's disease (Nerem and Sambanis, 1995).

.

Product	Wound market	Structure	Cost	Regulatory status	Refs.
Alloderm (LifeCell The Woodlands, Texas)	Severe burns Revisionary surgery Chronic skin ulcers Can be used when donor sites are limited	Cadaver skin with epidermis and dermal fibroblasts removed. The remaining dermal extracellular matrix is freeze dried and stored at either room temp or refrigerated until transplanted. Lack of cells make it nonimmunogenic and can be covered by patient's own autograft.	1995 price: $10K per square foot. 1994 sales $ 94K 1995 1st half sales $228K	On market Did not require FDA approval since it does not contain allogenic cells.	Szoka (1993) Glaser (1995)
Integra™ (Integra Life Sciences Corp. Plainsboro, NJ)	Full thickness burns, deep, partial thickness burns or where conventional autograft not available or desirable	Temporary treatment whilst autograft prepared. Bovine tendon collagen and chondroitin-6-sulphate matrix. Covered by a silastic layer which prevents water loss and protects from infection. Silastic layer removed after 14 days and replaced with patient's own autograft. Collagen portion degrades over 30 days. No rejection seen. Sold as sheets 10 x 25 cm, refrigerated in 70 % isopropyl alcohol	1996 price: $2K per square foot.	FDA pre-market approval March 1996. 1996 sold to Hong Kong, Singapore, Switzerland, Denmark and Ireland. Currently on market to trained personnel.	Cooper and Spielvogel (1994) Integra Life Sciences Corp. (1996) Development: Yannas and Burke (1980) Yannas *et al.* (1980)
Biobrane (Winthrop Labs. Santa Ana, CA)	Skin graft donor sites, partial and full thickness burns	Temporary covering. Nylon mesh with porcine peptides and thin layer of silicone. Peptides increase vascular ingrowth and adherence, whilst silicone is water vapour permeable and minimises bacterial infection. Allografts are preferred to Biobrane for treatment of large wounds.	-	On market and one of the most commonly used temporary coverings in 1994.	Cooper and Spielvogel (1994) Hansbrough *et al.* (1994)

Table 1.3. Continued on p.29

Dermagraft-TC (Advanced Tissue Sciences, La Jolla, CA. Smith and Nephew, London)	Transitional covering for severe burns	Cultured human neonatal foreskin fibroblasts seeded on a biodegradable scaffold. Scaffolds are made from suture materials, e.g.: polygalactin or poly glycolic acid and will be resorbed after 60-90 days. Covered by a semi-permeable membrane which is removed and replaced with an autograft. Sold in sterile packs of 5 x 7.5 inch sheets and are stapled onto the wound.	1995 price: $ 3K per square foot.	Received expedited review status in 1995. FDA approval granted 1996 The endpoint of the trial looked at the suitability of the wound bed for autograft take. Results were similar to those for temporary allograft application but secondary endpoints such as ease of removal and surgical satisfaction prompted approval	Cooper and Spielvogel (1994) Szoka (1993) Glaser (1995)
Graftskin now Apligraf® (Organogenesis, Canton, MA)	Permanent skin equivalent for treatment of burns, chronic and acute wounds.	Dermal equivalent consists of bovine type I collagen seeded with human neonatal foreskin fibroblasts. Epidermal layer provided by allogenic keratinocytes. Total manufacturing time 17-20 days. Cells originate from screened tissue and the epidermis appears to be immunologically inert. Appears to vascularise well.	-	FDA approval for treatment of venous ulcers in 1998.	Nerem (1992) Cooper and Spielvogel (1994) Eaglstein and Falanga (1997) Development: Bell *et al.* (1981) Wilkins *et al.* (1994)

Table 1.3. *Summary of details of dermal equivalents and skin substitutes. Data was collated from a number of sources and where available the selling price of the product is given.*

1.2. Plasma fractionation

Collected blood donations are centrifuged to produce three fractions, red blood cells, platelets and plasma. Between 1940 and 1942, Cohn developed an ethanol fractionation process for the extraction of proteins from plasma, initially removing albumin from the plasma for use in military medicine. In 1946, the process was developed for civil use and Cohn fractionation is still used to process most of the plasma throughout the world. The method involves adding varying concentrations of ethanol and adjusting the pH and ionic strength of the plasma to precipitate out proteins with varying solubilities. The first fraction precipitated contains fibrinogen, the least soluble of plasma proteins, clotting factor VIII, fibronectin and factors of the complement system. The second and third precipitated fractions contain immunoglobulins whilst the fifth fraction contains albumin (Kistler and Friedl, 1980). Further purification of each fraction releases the individual plasma proteins.

Plasma collected and separated from red blood cells can be fast frozen and stored at -40°C until required. The least soluble proteins in plasma form the cryoprecipitate when plasma is frozen and they are prevented from redissolving on thawing by maintaining the thaw temperature at less than 4°C. The thawed plasma is centrifuged so that the least soluble proteins are collected as a precipitate. This cryoprecipitate contains approximately 60 % of both the fibronectin and Factor VIII present in the initial plasma but the main protein component is fibrinogen. Other proteins such as vonWillebrand's factor are also present and can be extracted for therapeutic purposes. The main commercial product extracted from cyroprecipitate is Factor VIII. Example schemes for the extraction of fibronectin from Factor VIII cryoprecipitates are shown in Figure 1.1.

Other precipitants such as ammonium sulphate, Rivanol® and caprylic acid have been used in combination with each other and with chromatography steps to extract proteins difficult to separate by ethanol fractionation (Steinbuch, 1980).

1.2.1. Plasma fractionation with polyethylene glycol

Polyethylene glycol (PEG) was first used as a precipitating agent in 1956 and has advantages over ethanol in plasma fractionation in that it is non-toxic, non-flammable and non-denaturing and can be used at temperatures above 0°C to fractionate proteins (Hao *et al.*, 1980). It acts by removing the water available for proteins to remain in solution and thus increases the effective concentration of each protein present. It was used to purify factor VIII, albumin and other plasma proteins on a large scale in the 1970s (Hao *et al.*, 1980). Hao *et al.* also describe fractionation experiments with Factor VIII-depleted cryosupernatant. Addition of increasing amounts of PEG resulted in extraction of smaller and more soluble proteins:

0 - 4 % PEG precipitate - fibrinogen

4-10 % PEG precipitate - plasminogen, components of complement, immunoglobulins and β-lipoproteins

10-20 % PEG precipitate - prothrombin, α_2-macroglobulin, IgA, coagulation factors

> 20 % PEG precipitate - albumin, transferrin, ceruloplasmin and haptoglobin.

Final chromatography steps allowed extraction of the proteins in a more native form than with ethanol.

Although PEG is non-toxic, it is preferable to remove all chemicals added during purification. PEG has no charge and thus can be removed by ion exchange or affinity chromatography or can be separated by 2 phase aqueous partition. These can all be carried out on a large scale (Hao *et al.*, 1980).

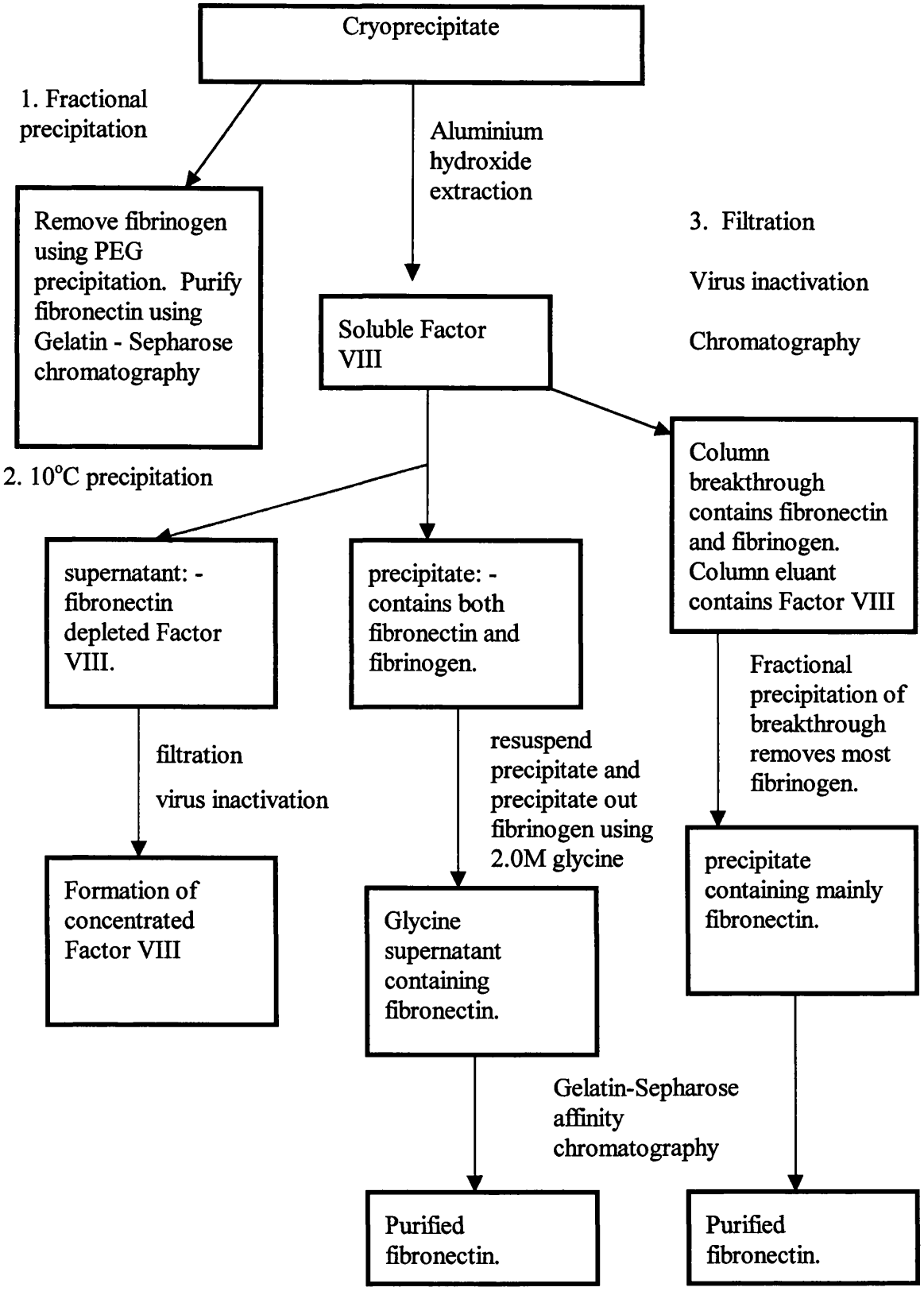


Figure 1.1. *Overview of potential methods for extracting fibronectin from a cryoprecipitate fraction of plasma. Method 1 would be suitable for lab scale operations only as no clotting factors are recovered. Methods 2 and 3 involve the extraction of fibronectin and fibrinogen fractions in established Factor VIII production processes. In depth process details are not provided. (Adapted from Horowitz et al., 1984 and Horowitz and Chang, 1989, also information from PFC, Edinburgh)*

1.3. Fibronectin

Fibronectins are a class of high molecular weight glycoproteins found in two distinct forms. Plasma fibronectin is secreted into the blood stream by hepatocytes whilst the cellular or insoluble form is produced by fibroblasts and remains localized on the cell surface. The soluble form of fibronectin is also found in most body fluids.

1.3.1. History and Isolation

In 1948, Morrison *et al.* observed, during further purification of a fibrinogen rich plasma fraction, an unrecognised component which had a lower solubility than fibrinogen at low temperatures. Accordingly this component was named 'cold-insoluble globulin'.

A pure fraction of cold-insoluble globulin was not made until 1970, when Mosesson and Umfleet (1970) used diethylaminoethyl cellulose chromatography to remove fibrinogen and other plasma proteins from cryoprecipitate. Confirmation that the cold-insoluble globulin had an electrophoretic migration rate similar to a fast β globulin and that no change was noted after heating to 56°C or treatment with thrombin distinguished it from fibrinogen and it was then regarded as a separate plasma protein.

Other research during the 1970s concentrated upon the isolation of a cell surface protein from fibroblasts (Hynes 1990). Yamada and Weston (1974) isolated a protein, present on the surface of normal cells but absent after neoplastic transformation. This cell surface protein (CSP) was seen to increase with cell density and deemed important in growth control. In the mid-1970s it was concluded, by a number of groups, that the cell surface protein and cold insoluble globulin were related with properties including cell adhesion and morphology. Antibodies were used both to confirm similarities between the two proteins (Hynes 1990) and some slight structural differences (Atherton and Hynes, 1981). Gelatin affinity chromatography was described as a rapid and simple method to purify proteins from both sources (Engvall and Ruoslahti, 1977).

Research into both soluble and insoluble forms of the protein and their properties had been carried out independently resulting in a number of different names for the protein each describing its identified functions. The protein had been described as cold-

insoluble globulin, antigelatin factor, cell attachment protein, cell spreading factor and opsonic α_2 surface binding glycoprotein whilst the cell surface forms had been known as large, external, transformation-sensitive (LETS) protein, surface fibroblast antigen, galactoprotein a, cell surface protein and zeta protein. To avoid further confusion it was decided that the proteins should be described as cellular and plasma forms of fibronectin, derived from Latin *fibra* - fibre and *nectere* - to connect or link, referring to the adhesion properties of the protein (Hynes, 1990).

By 1982 a fairly clear picture of the basic structure and functions of the molecule was established and with the advent of molecular biology, by 1988, complete amino acid sequences of a number of mammalian fibronectins were known as well as a more detailed knowledge of the protein structure (Hynes, 1990).

1.3.2. Primary structure and conformation

Fibronectin is a dimer, of molecular weight approximately 440 kDa, consisting of two almost identical chains which are discretely separable on an electrophoretic gel. Estimates of the molecular weight of each chain have been given as 220 and 215 kDa and this difference is attributed to the release of a small C terminal peptide post-translationally rather than the synthesis of two types of chain (Mosesson *et al.*, 1975). The chains are covalently joined by disulphide bonds at their C termini, as shown in Figure 1.2. At the N terminus there are a number of intrachain disulphide bonds and in the central portion of each chain 1 or 2 free sulfhydryl groups are found as well as the carbohydrate residues, which are believed to stabilise the protein against proteolysis (Mosesson and Amrani, 1980).

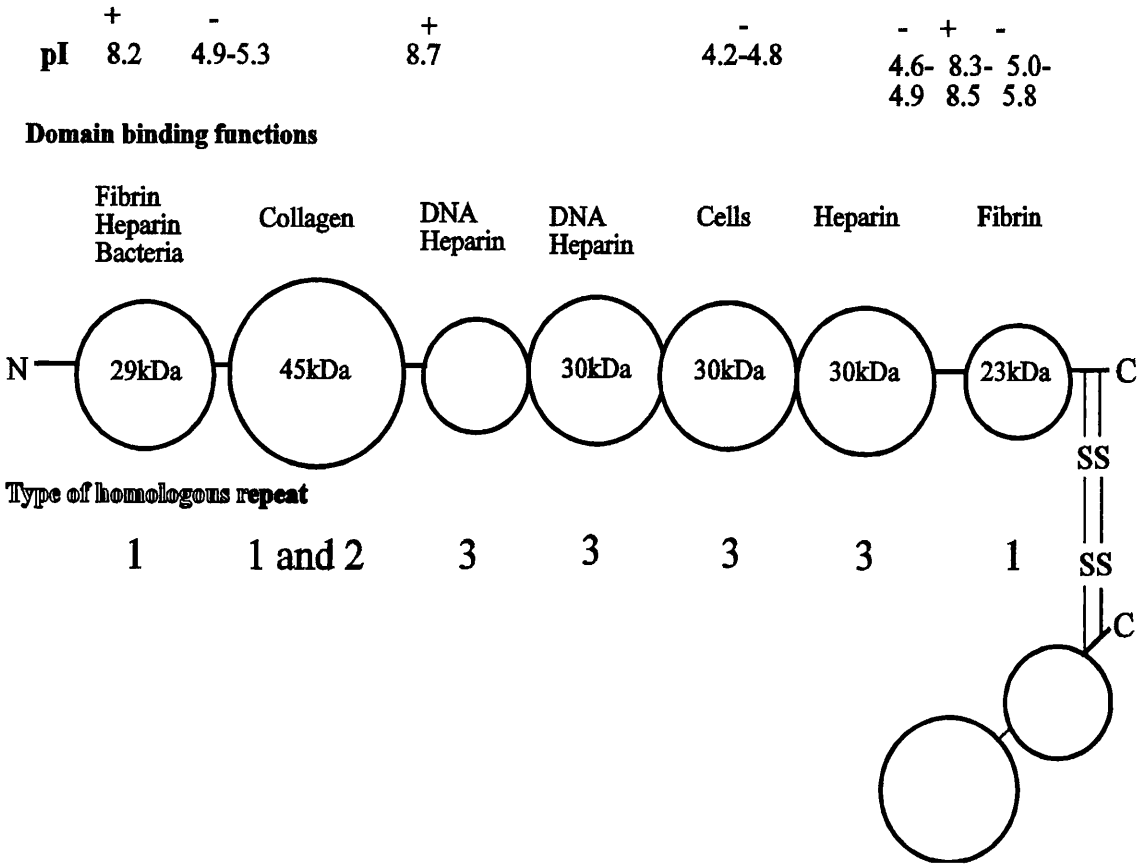


Figure 1.2. *Schematic diagram of the fibronectin molecule. Fibronectin consists of two almost identical chains, one shown here completely and one partly shown, joined at their C termini by disulphide bonds to give a flexible structure. Each chain is divided into a number of domains, each with particular binding functions, shown above the diagram. Approximate molecular weights for each domain are given, with the rest of the molecular weight of the chain (total 215 - 220 kDa) being made up by connecting sequences. 90 % of the fibronectin molecule is made up of 1 of 3 repeat amino acid sequences and the type of repeat predominant in each binding domain is shown below the chain. The pI for individual sections of the chain was found to differ and these are also shown in approximate positions along the chain as well as the ionic charge for that region at neutral pH (adapted from Markovic et al, 1983b and Petersen et al., 1989).*

The two chains, each approximately 2000 amino acids long, are separated at physiological pH by a 70° angle (Mosher, 1984). The position of the chains under various environmental conditions is described more fully is section 1.3.3.

The fibronectin molecule was found to consist of a number of homologous repeat sequences, listed below:

Type 1: 45-50 amino acids long and containing disulphide bonded loops

Type 2: as type 1

Type 3: 90 amino acids long but with no disulphide bonds.

The central 150-170 kDa portion contains only type 3 repeats and thus no disulphide bonds, although the two free sulfhydryl groups are found here. Each repeat exists as a basic binding module and so repeat sequences increase the specific binding potential of each domain. The cell binding domain was seen to contain a number of repeats of the four amino acids, L-arginine, glycine, aspartate and serine or closely related variants and these are known to promote the cell attachment and cell spreading functions of fibronectin (Hynes, 1985).

Boughton and Simpson (1984) examined affinity chromatography purified fibronectin which appeared homogeneous by SDS gel electrophoresis but could be split into five fractions by isoelectric focusing, each with a different isoelectric point (pI) ranging from 5.6- 6.1. The fraction with pI = 6.1 was the only fraction found to promote the ingestion of *Sacchromyces cerevisiae* and constituted only 15 % of the total fibronectin. They also found that fibronectin in plasma had a higher pI than purified fibronectin although the explanation for this was unclear. Markovic *et al.* (1983b) found that different regions of the chain had varying values of pI and this may play an important role in the conformation of the molecule and its binding functions described later. Tooney *et al.* (1983) have measured the pI of a 6.8 mg/mL solution as 5.2 ± 0.2.

It is known that there is only one gene for fibronectin (Petersen *et al.*, 1989) and that different types of fibronectin are formed after being transcribed in a different way. It is as yet unknown whether different fibronectins originate from different tissues or whether they interact with different molecules preferentially (Boughton and Simpson, 1984).

1.3.3. Effect of environmental change on the fibronectin molecule

The effect of various environmental conditions such as pH, temperature, ionic strength and chemical agents has been examined thoroughly to determine whether they effect the conformation of the molecule. Changes in molecule shape may affect the binding properties of the protein.

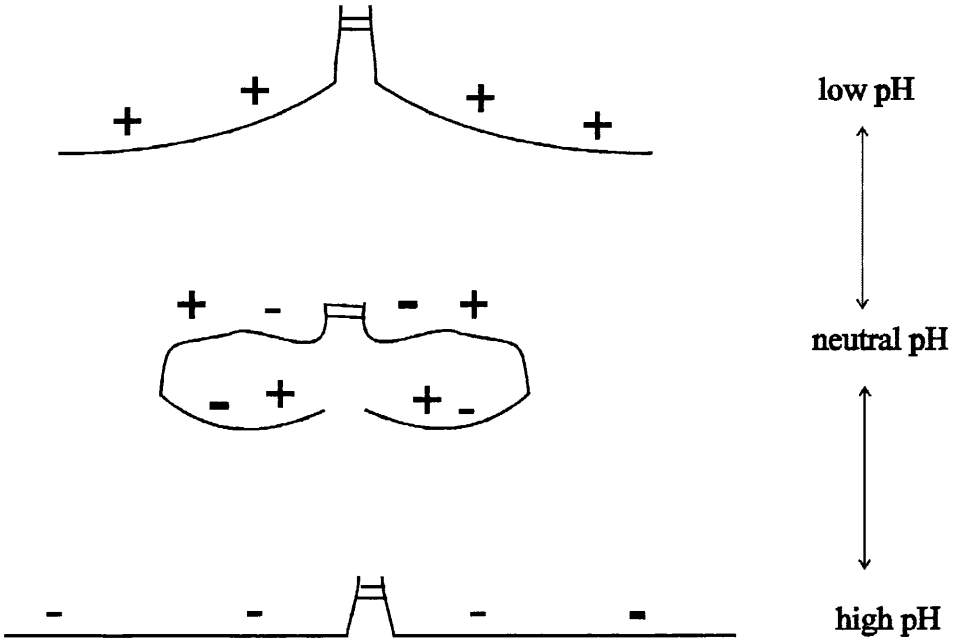
1.3.3.1. Effect of pH and ionic strength

Electron microscopy has been used to measure the dimensions of the fibronectin molecule. Most accurate results are believed to be achieved when fibronectin is freeze

dried on a carbon film at pH 7.0. Under these conditions, Tooney *et al.* (1983) measured the molecule as 24 nm x 16 nm.

There have been a number of reports suggesting that the fibronectin molecule changes from a compact to an elongated conformation under extremes of pH and ionic strength. Tooney *et al.* (1983) noted that the protein unfolds at pH 2.8 or 9.3 or when the ionic strength is raised at pH 7.0. Similar results were seen by Williams *et al.* (1982) who described an increase in chain flexibility and Sjoberg *et al.* (1989) who stated that fibronectin molecules in solutions with sodium chloride concentrations <0.3 M are disc-shaped, whilst in 0.3 M solutions they take on a reversible transition to a more open structure.

A number of studies have been made to investigate changes in electrostatic bonding in the molecule with changes in the environment. A study by Markovic *et al.* (1983b) indicated, using sedimentation velocities, that fibronectin molecules formed elongated structures with 'outstretched arms' at pH 3.0 and 11.0. To test whether the effect was due to intra or inter chain effects a 140 kDa fragment from the central portion of one chain was also tested. A similar pH dependent effect was seen. Small segments taken from the N terminal end of the chain showed no pH dependence on sedimentation velocity. These may have been stabilised by disulphide bonds in the region. The group also noted that the sedimentation velocity for pH 6-10 alluded to a compact structure and a rod-like form for pH < 6.0, concentrations 0.15 mg/mL. This is represented diagrammatically in the upper part of Figure 1.3. The pH-dependent reaction is believed to be brought about by interactions between the domains of the molecule. Electrostatic interactions between domains of different net charges (due to differing pI in adjacent segments along the chain, Figure 1.2) were believed to be important for intramolecular association. At neutral pH the opposing charges interact to produce a fairly symmetrical compact conformation whereas at extremes of pH a more extended, thread-like conformation forms due to repulsive interactions.



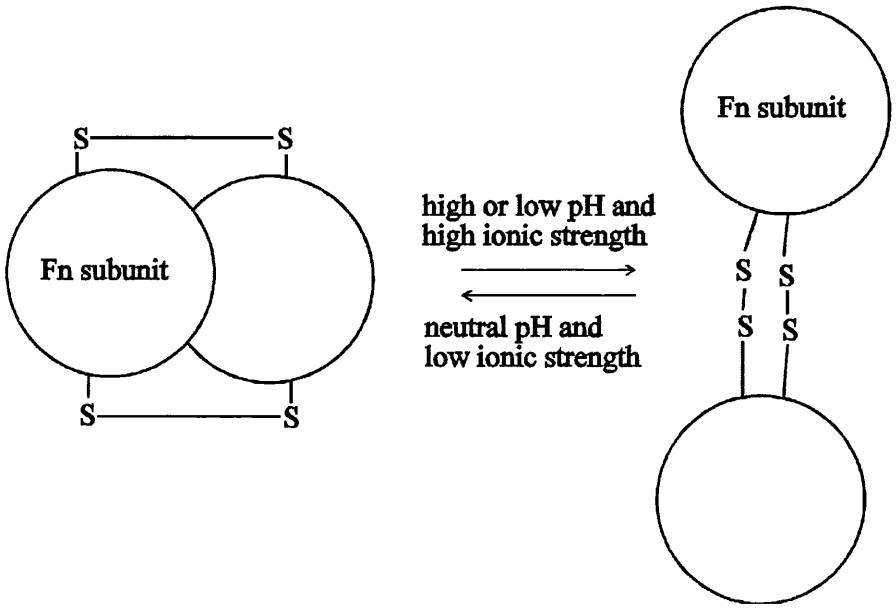


Figure 1.3. *The effect of environmental change on the conformation of the fibronectin molecule. Above - the view of Markovic et al. (1983b). The molecule forms a compact, symmetrical shape at neutral pH due to the arrangement of opposing charges on each chain. Alteration of these charges by changes in environmental conditions leads to repulsion and a thread-like structure. Below - from Benecky et al. (1991).* Each *disk represents one chain of the fibronectin dimer, joined to another disk by disulphide bonds. Under physiological conditions the disks are compacted due to the attraction of opposite charges. Changes in the environment disrupt bonds between the two chains and the disks are free to rotate about their C termini.*

More recently, Benecky *et al.* (1991) have compared the relative importance of intrasubunit and intersubunit electrostatic forces by comparing dimeric fibronectin with a 190 / 170 kDa fragment from one chain. Sedimentation velocities of dimeric fibronectin at pH 2.0, 11.0 or increased ionic strength decreased whilst those for the fibronectin fragment were relatively insensitive. These results confirm unfolding of the molecule at pH 2.0, 11.0 and increased ionic strength but attribute this to salt or pH altering interchain electrostatic interactions / hydrogen bonds with minimal alteration to the intrasubunit bonding (Figure 1.3 -lower).

There is a general agreement that the molecule undergoes a transformation from compact to open formation but Markovic *et al.* described a thread-like molecule with length 120-140 nm, when unfolded, caused by disruption to intrachain bonding whilst Benecky *et al.* discussed the formation of two disks, each of diameter 20 nm and thickness 2.3 nm, which could independently rotate about their C termini.

1.3.3.2. Effect of temperature

Changes are believed to occur to the fibronectin molecule at temperatures both above and below body temperature. When Brown *et al.* (1987) used monoclonal antibodies to determine whether fibronectin underwent a conformational change between 37 and 4°C he found three antibodies bound more tightly to fibronectin at 4°C. Sedimentation velocities pointed to less extensive intramolecular rearrangements than occur at extremes of pH and ionic strength. The changes which occur on cooling may also involve the domains associated with fibronectin self-aggregation and those associated with fibrin-heparin-fibronectin cryoprecipitation. Tooney *et al.* (1983) noted enhanced self-aggregation of fibronectin fibrils at low temperatures and protein concentration 1-7 mg/mL.

Miekka (1983) noted fragmentation of fibronectin structure on heating samples to 100°C for 2 minutes and extra bands were visible on SDS electrophoretic gels between molecular weights 10 and 200 kDa with particularly obvious bands at 65 and 100 kDa. Such fragmentation was seen at 70°C but not at 60°C when fibronectin was heated. No fragmentation of fibrinogen was noted under these conditions. This difference was attributed to the domain structure of fibronectin which exposes regularly spaced labile bonds for cleavage. Alexander *et al.* (1979) and Miekka (1983) noted that

fragmentation on heating was accelerated under acidic conditions. In contrast, Osterlund (1988) stated that secondary fibronectin structure was preserved between 20 and 70°C, even at pH 3.0, and that heating induced conformational changes were reversible up to temperatures of 55°C.

1.3.3.3. Effect of binding to other ligands

An increase in chain flexibility was seen on binding of collagen (Williams *et al.*, 1982) and heparin (Tooney *et al.*, 1983; Khan and Newman, 1991) to their specific binding sites.

1.3.3.4. Effect of denaturing agents

Different regions of the fibronectin molecule exhibit varying sensitivities to urea (Markovic *et al.*, 1983) and guanidine hydrochloride (Khan *et al.*, 1990). In both cases the disulphide-rich N-termini ends were found to be more stable than the more central regions. Some regions were irreversibly affected by 4 M urea at 4°C, which highlights a problem in gelatin affinity chromatography where fibronectin is routinely eluted from the column with 4-8 M urea. Under strong guanidine hydrochloride denaturing conditions the molecule was believed to become completely unfolded and lose all its secondary structure.

1.3.4. Fibronectin in plasma

Concentrations of fibronectin in human plasma are approximately 220-300 μg/mL (Reilly *et al.*, 1983; Snyder *et al.*, 1984; Perttila *et al.*, 1990). It is believed to serve an opsonic function in the plasma and depleted plasma levels have clinical significance as a build-up of particulate debris can occur in the circulatory system. Patients with low fibronectin concentrations have been shown to have reduced resistance to infection and infusion of antifibronectin reduces survival from shock, trauma and burn injury (Reilly *et al.*, 1983).

Fibronectin is synthesised and secreted in large amounts by a number of cell types but predominantly hepatocytes and has a half -life in the plasma of 24-72 hours (Mosher, 1984). It has been suggested that cellular fibronectin may be able to undertake the role of plasma fibronectin when plasma levels are depleted and in return, the dimeric plasma

fibronectin is also known to incorporate itself into the pool of insoluble fibronectin, following the formation of multimers (Saba and Jaffe, 1980).

1.3.5. Biological properties of fibronectin

Fibronectin performs a major role in a variety of activities including substrate adhesion, cell spreading, alignment and morphology as well as an opsonic function in the plasma. These functions are modulated by the affinity of fibronectin for a number of molecules found in the extracellular matrix, including collagen, fibrin and fibrinogen, heparin, actin and DNA (Mosesson and Amrani, 1980; Yamada, 1989). The areas of the molecule with specific binding capabilities are shown in Figure 1.2. Cell adhesion to fibronectin will be discussed in detail in section 1.5. The binding of some of the ligands is highly specific, e.g.: binding of heparin does not affect the cell binding domain, whereas some of the domains are less specific and other domains can be blocked by steric interference if a large molecule is bound to one site (Yamada, 1989).

1.3.5.1. Fibronectin in the extracellular matrix

Soluble plasma fibronectin can undergo fibrillogenesis to form multimers of the insoluble form of fibronectin. Although fibronectin fibrillogenesis has been noted in stored solutions and is believed to involve electrostatic interactions between the protein dimers, the fibronectin in the extracellular matrix is in the form of covalently bound aggregates. A different method is hypothesised for the formation of these.

Fibronectin - fibronectin self assembly

Fibronectin has been seen to assemble into aggregates which resemble the protein fibrils in the extracellular matrix after storage at 4°C (Vuento *et al.*, 1980). An examination of the fibrillogenesis of fibronectin by Homandberg (1987) showed that the N terminal 29 kDa fragment was involved in fibronectin-fibronectin binding and that electrostatic interactions occurred between oppositely charged N and C termini. Under physiological conditions the molecule exists as a 'V' shape, making fibril formation difficult, but electrostatic interactions can occur when the molecule is in its extended conformation (Hynes, 1990). Due to the absence of disulphide bonds at the C terminus no disulphide

exchange occurs and the formation of these fibrils can be disrupted by high salt concentrations.

Cell surface receptor mediated matrix assembly

The wide ranging binding properties of the fibronectin molecule allow it to act as a scaffold for the organisation of components of the extracellular matrix (McKeown-Longo and Mosher, 1989). Experiments using plasma fibronectin have identified a scheme for fibronectin incorporation into the extracellular matrix although it is not clear whether cellular fibronectin would act in a similar manner.

The N terminal portion of the fibronectin molecule binds to a cell surface receptor in an orientation which facilitates both fibronectin-fibronectin interactions and disulphide exchange between N terminal regions. Once bound, a fibronectin molecule has a 0.2 % probability of being assembled into the matrix in one minute. Binding to the receptor is reversible and once the growing fibril is complete and disulphide exchange has occurred, the fibril is released and the receptor is recycled. Fibronectin multimerisation can also occur due to Factor XIII (transglutaminase) cross-linking when the fibril is bound to a cell surface receptor (McKeown-Longo and Mosher, 1989; Fogerty and Mosher, 1990). The final matrix consists of covalently bound, insoluble fibronectin which can then bind other extracellular matrix components, such as collagen, as well as attaching to the cells.

1.3.5.2. Superfibronectin

Superfibronectin is a form of the protein with improved cell adhesion properties. A recombinant fragment, resembling a type III repeat involved in fibronectin - fibronectin binding, is produced and allowed to bind to fibronectin. As a result spontaneous cross-linking of the molecule occurs and multimers are formed which are similar in mass to cellular fibronectin matrix fibrils. This new form of fibronectin has been shown to have enhanced cell adhesive properties and can suppress cell migration. Cell-fibronectin interactions occur not only through the traditional fibronectin binding receptors, integrins, but also through another set of non-related receptors. Pretreatment of tumourogenic cells with this new superfibronectin may reduce cell migration and thus have an antimetastatic effect (Moria *et al.*, 1994; Pasqualini *et al.*, 1996).

1.4. Fibrinogen

Fibrinogen is the soluble precursor to fibrin, a thread-like material, essential for blood clotting. It is synthesised in the liver and found in the plasma at concentrations 2-4 mg/mL and is one of the least soluble plasma proteins, making it easy to precipitate with salts such as sodium chloride and ammonium sulphate as well as being precipitated at low ionic strength solutions. During plasma fractionation, it is frequently extracted using the cold ethanol method with different fractions possessing varying clotting properties. The isoelectric point has been measured as 5.5 (Doolittle, 1975).

1.4.1. Structure

The molecular weight of fibrinogen has been measured as 340 ± 20 kDa and it is known to consist of 3 pairs of non-identical chains, joined by disulphide bonds. The three sets of chains, Aα, Bβ and γγ all have different amino acid compositions and have molecular weights of 65.5, 56 and 47 kDa respectively. Like fibronectin, fibrinogen is a glycoprotein and is believed to take on an elongated conformation at extremes of pH and high ionic strength. However the exact conformation of the molecule at physiological pH is debated. The favoured structure consists of 3 domains joined by linking sequences, shown in Figure 1.4. The central domain consists of the N terminal domains for all 6 chains whilst the larger end domains contain C termini. Fibrinogen has no free sulfhydryl groups but contains 21 - 34 disulphide bonds, with almost 50 % of these bridges confined to the N terminal forming a 'disulphide knot'. The N terminus of the Aα chain contains the 16 amino acid long fibrinopeptide A, whilst the Bβ chain contains the 14 amino acid long fibrinopeptide B.

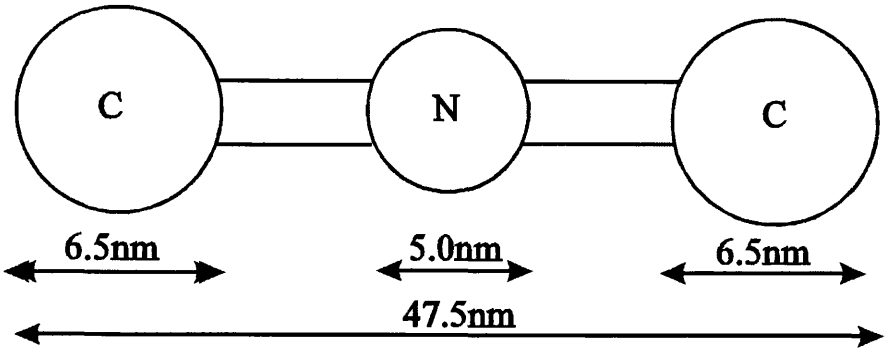


Figure 1.4. *Schematic diagram of fibrinogen structure. The cluster of disulphide bonded N termini forms the 'disulphide knot'. Electron microscopy, enzymatic degradation studies and sedimentation and diffusion data support this structure. It is believed that the domains are flexibly linked and independent (Doolittle, 1975).*

1.4.2. Fibrinogen's role in blood clotting

The most important role of fibrinogen, *in vivo,* is that of blood coagulation although it is also believed that fibrinogen may play a part in defence against infection. In the blood clotting cascade fibrinogen is transformed into insoluble fibrin by the serine protease thrombin. Fibrin has a very similar structure to fibrinogen with only the four fibrinopeptides, two A and two B, at the N termini of the A and B chains respectively being cleaved by thrombin to form the clotting protein. In the complete fibrinogen molecule opposite ends of the chains have opposing charges, with the central portion having an overall negative charge, under physiological conditions. Cleavage of the negatively charged fibrinopeptides from the N terminus results in both N and C termini having a positive charge. As a result the fibrinogen monomers form a staggered overlapped structure, similar to a brick wall, where the positively charged end of the molecule forms an electrostatic interaction with the centre of another monomer. These monomers are then stabilised by a cross-linking agent, Factor XIII, present in plasma in a non-activated form but transformed into an active state by thrombin and calcium ions. When active it covalently links lysine and glutamine residues to form up to 6 bonds per dimer, which serve to stabilise the polymerised fibrin and form a clot. Fibrin clots can be broken down after they have served their purpose by another enzyme, plasmin, which exists in plasma in its inactive form, plasminogen (Doolittle, 1975).

1.4.2.1. Fibronectin's role in blood clotting

From the earliest isolation of fibronectin in 1948 it has been known that the protein interacts with fibrinogen. Fibronectin modulates blood coagulation using its affinity

for fibrinogen to become incorporated into fibrin clots as well as its ability to interact with fibrin, cells and other proteins such as collagen and it has been shown that fibronectin is the major non fibrin protein of the clot.

Fibronectin interacts with the polymerising fibrin, mainly via a binding site near the N terminus of the fibronectin, cross-linking the protein to the C terminal end of the fibrin α chain. It does not significantly inhibit clot formation by preventing fibrin-fibrin interactions, instead it plays an important role in cell attachment to the clot and has been shown to increase fibroblast adhesion and migration into clots. Fibronectin is also known to interact with platelets, granulocytes, fibroblasts, endothelial cells and mononuclear phagocytes associated with the clot.

Fibronectin has a number of antithrombotic actions including inhibition of platelet aggregation and promotion of attachment and spreading of platelets on collagen surfaces. Combined with these fibronectin also enhances solubilisation of fibrin complexes and fibrin-collagen complexes and removal of fibrin-fibrinogen-fibronectin complexes from the vasculature by attracting cells to remove the debris (Kaplan and Snedeker, 1980; Moon and Kaplan, 1989).

Fibronectin is a substrate for Factor XIIIa and can be incorporated into a fibrin clot by covalent cross-linking (Mosher, 1975). Studies by Carr *et al.* (1987) with fibrin gels suggested that fibronectin alone had no impact on fibrin assembly but on addition of Factor XIIIa an increase in fibrin fibril diameter and density within the gel was seen. As the fibres become larger, the spaces between the gel also become larger allowing the diffusion of small proteins such as albumin and IgG. They concluded that the addition of fibronectin to fibrin in the presence of Factor XIIIa resulted in a network of large, dense fibres and could influence the structure of clots. Procyk *et al.* (1985) also studied gel formation and hypothesised that fibrinogen-fibronectin heteropolymers were formed first and increased in size to form particulate matter but that the main structure of the gel was formed by fibrinogen homopolymers. The fibronectin was incorporated either as a monomer or as heteropolymers by Factor XIIIa.

1.4.3. Other interactions between fibronectin and fibrinogen

Interactions between fibronectin and fibrinogen / fibrin in the blood clotting cascade have been discussed in section 1.4.2. and the importance of their interrelationship highlighted. This section describes interactions essential for protein precipitation at low temperatures. There are two forms of fibronectin - fibrinogen precipitates which can be formed from plasma in the cold. The first, induced by a sulphated mucopolysaccharide such as heparin, is described as the 'heparin precipitable fraction' whilst the second, requires no heparin (Mosesson and Amrani, 1980).

Heparin forms precipitable complexes in the cold with fibronectin but not fibrinogen alone, although addition of fibrinogen reduces the threshold for heparin induced precipitation and increases the amount of precipitate formed. Fibronectin forms soluble complexes with heparin at 22°C which precipitate on cooling to 2°C (Stathakis and Mosesson, 1977). Discrete binding sites for fibrinogen and heparin on fibronectin allow all three proteins to be incorporated into the precipitate. Fibrinogen molecules lacking their C termini of the Aα chains, the region known to bind to fibronectin, failed to become incorporated into heparin-induced cryoprecipitates.

Stathakis *et al.* (1978) investigated the cryoprecipitation of fibronectin, fibrinogen and fibrin in the absence of heparin. They found that fibrinogen columns bound fibronectin at 4°C but very little bound at 22°C but that fibrin columns bound significant amounts of fibronectin at both temperatures. Testing the precipitation of the three components, they discovered at 2°C, neutral pH and low ionic strength that fibronectin or fibrinogen alone or as a mixture did not precipitate out. Fibrin and fibrinogen mixtures under these conditions did not precipitate out unless fibronectin was added. Fibrin - fibrinogen complexes lacking the C terminus fibronectin binding site failed to precipitate. It was therefore concluded that both fibrin and fibronectin were necessary for this non-heparin precipitation and that fibronectin acts as a nucleus with its multiple binding functions for the fibrin - fibrinogen complexes.

1.5. Wound healing and contact guidance

In order to be able to successfully heal wounds and replace tissue, an understanding of the natural process is required. A brief description of wound healing is given in section 1.5.1 describing the importance of fibrinogen and fibronectin in the process. Wound contraction is an essential feature in order to bring the wound margins closer together. However it can be exaggerated and result in scar formation or, if in the region of a joint, loss of mobility.

1.5.1. Wound healing and the role played by fibronectin and fibrinogen

Due to the accessibility of the skin, much of the study of wound healing has been carried out on cutaneous wounds and it is the healing of a skin wound which is now described.

Immediately after wounding, damaged blood vessels release chemotactic factors and plasma proteins, including fibronectin and fibrinogen, as well as clotting factors required for blood coagulation. A fibrin blood clot forms (1.4.2.) containing fibronectin and cross-linked by Factor XIIIa to reduce blood loss through the wound. Platelets in the fibrin clot secrete growth factors to promote fibroblast migration. Minutes after the injury neutrophils and monocytes respond to the chemotactic signals of, amongst other peptides, fibronectin. Neutrophils are responsible for management of infection whilst monocytes remove tissue debris and secrete growth factors. In turn, macrophages are recruited into the area in order to phagocytose fibronectin coated debris and bacteria. The fibronectin content of the wound matrix is higher than that of adjacent tissue.

Several hours after wounding, keratinocytes in the edges of the wound produce new receptors which allow them to crawl over the wound margin and initiate the process of re-epithelialization. During the stages of epithelilazation, cell movements will be blocked by the presence of the fibrin clot. The migrating keratinocytes produce the tissue-type or urokinase type plasminogen activator which converts the inactive plasminogen, released from the clot, to active plasmin, leading to fibrinolysis. Other matrix metalloproteases are up regulated by the migrating keratinocytes and are responsible for the breakdown of other matrix proteins, allowing keratinocytes to migrate over the wound. It has been shown that high levels of proteolytic activity are present in wound fluids which may be a reason why they are difficult to heal. Fibronectin acts as a substrate for keratinocyte

migration, whilst numerous growth factors released at the site of injury stimulate keratinocyte migration.

Skin fibroblasts are usually quiescent in non-wounded tissue. They are activated by numerous chemotactic factors, such as platelet derived growth factor (PDGF) and transforming growth factor α (TGFα), which causes them to upregulate their fibrin, fibronectin and fibrinogen receptors. The fibroblasts then proliferate and use fibronectin in the clot material to migrate into the wound, invade the clot and align themselves using fibronectin-rich fibrils. In the clot fibroblasts use fibronectin as a scaffold to lay down a collagen rich matrix, 'granulation tissue', which loosely surrounds the cells. Fibronectin gradients in the wound also promote the ingrowth of endothelial cells which will revascularise the area. After one week the clot is fully invaded and replaced by activated fibroblasts. The new granulation tissue is rich in fibronectin and collagen type III although with time these are replaced by collagen type I and the new tissue is remodelled by fibroblasts. The fibroblasts secrete proteolytic enzymes to break down collagen and use fibronectin again as a template for deposition of new material. If the final tissue does not resemble the original collagen network, then scarring may result.

Whilst the granulation tissue is formed, fibroblasts at the edges of the wound start to bring the edges of the wound together by wound contraction. It is hypothesised that fibroblasts involved in this process take on elements of smooth muscle cells and are termed 'myofibroblasts'.

Endothelial cells migrating into the wound upregulate integrin receptors so that they can respond to chemotactic signals, such as fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF). New blood vessels are formed to supply the new tissue (Grinnell, 1984; Clark, 1988; Colvin, 1989; Grinnell 1994; Martin, 1997).

1.5.2. The fibronectin receptor

Fibronectin acts as a nucleating agent, binding cells to substrates such as collagen, fibrin and glycosaminoglycans. Fibroblasts, hepatocytes, mesenchymal, neural, epithelial and endothelial cells are amongst the many types of cell which have affinity for fibronectin (Yamada *et al.*, 1985; Kleinman *et al.*, 1987).

As described earlier, fibronectin's cell binding domain contains a number of repeats of the cell adhesion tripeptide, RGD (arginine, glycine, aspartic acid), which is known to bind to receptors on the surface of a number of cell types. Fibrinogen also contains this sequence (Ruoslahti and Pierschbacher, 1987). The receptors which recognise this RGD sequence are called integrin receptors and contain two membrane spanning subunits. Receptor binding requires divalent cations and results in transduction of signals across the membrane by interacting with the cell's actin cytoskeleton. Integrins are 140 kDa heterodimers consisting of non-covalently linked α and β subunits. In 1993, Brown and McFarland reported that 14 α and 8β subunits had been identified so far. Fibronectin can bind cells via 7 RGD dependent integrin receptors, one RGD independent integrin receptor, several non-integrin binding sites and a combination of the above.

Some integrins bind to RGD of single proteins whilst others are less specific. There are a minimum of 10 amino acid recognition sequences related to RGD and at least as many receptors compiling a versatile recognition system which provides cells with anchorage, traction for migration and signalling mechanisms for polarity, position, differentiation and possibly growth. Addition of RGD-like peptides to a cell system has been shown to inhibit cell adhesion to a fibronectin substrate (Ruoslahti and Pierschbacher, 1987). The fibronectin integrin receptor, $\alpha_5\beta_1$, is important in cell attachment and phagocytosis whilst $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_v\beta_3$ also bind fibronectin. The receptor $\alpha_v\beta_3$ is known to bind to fibrinogen (Greiling and Clark, 1997).

1.5.3. Cellular interactions with fibronectin and / or fibrinogen substrates

Cells adhere to fibronectin and fibrinogen substrates via the integrin receptors described above. The interactions of cells with fibronectin and fibrinogen substrata have been studied by a number of groups with implications for tissue engineering. Grinnell and Feld (1979) concluded that human skin fibroblasts secreted a factor onto a tissue culture substratum to aid their own spreading and that this factor was probably fibronectin. In 1980, Grinnell *et al.* noted that baby hamster kidney (BHK) cells would only attach to fibrinogen or fibrin in the presence of fibronectin. Cell adhesion and spreading was further enhanced when substrates were covalently cross-linked with Factor XIII in the presence of thrombin.

Donaldson and Mahan (1983) found that both fibronectin and fibrinogen substrata supported considerable epidermal cell migration in the healing of adult newt skin wounds. Migration could be prevented, in both cases, by the addition of the appropriate antiserum. It is however feasible that fibrinogen was converted to fibrin in the wound environment or that there was modification of the test surfaces by the wound fluids. In contrast, Corbett *et al.* (1996) determined that plasma fibronectin covalently linked to fibrin formed a functionally distinct surface from plasma fibronectin on a plastic surface and that spreading of NIH-3T3 cells reached only 50 % of their maximal spreading on the fibrin - fibronectin surface, compared to the fibronectin on plastic. Cells were found not to attach nor spread on cross-linked fibrin alone.

Palecek *et al.* (1997) have discussed the relationship between cell migration rate and cell binding to the substrata. At fibronectin concentrations lower than those giving rise to maximum speed cells are rounded but extend processes, which are shorter than usual and often too small to move the migrating cells. At higher ligand concentrations than those that give rise to maximal speed, cells have a highly spread morphology and extend lamellae similar to migrating cells but the cell body does not move well due to strong adhesions with the fibronectin substrate. Thus maximal cell migration speed involves balancing both fibronectin and receptor concentration as well as the affinity between them. Cell movement will be discussed in more detail in section 1.5.4.1.

The role played by fibronectin as a conduit for migration in wound healing was examined by Greiling and Clark (1997). They set up a system where fibroblasts, embedded in a collagen gel, were induced to migrate to a fibrin clot by platelet derived growth factor. Fibronectin was contained in both gel and clot. Removal of fibronectin from the either end of the system resulted in a decrease in the number of migrating cells suggesting that fibronectin was acting as a conduit for migration. If fibronectin was removed from the clot then there was an 80 % decrease in transmigration, which could be restored by addition of fibronectin. No transmigration could be seen when fibronectin was removed from the collagen gel and movement was inhibited when RGD peptides or monoclonal antibodies against the integrin receptors were added.

1.5.4. Cell movement and contact guidance

Theories describing contact guidance and orientated cell movement have been put forward since the early part of this century. However researchers have often found it difficult to prepare homogeneous testbeds to isolate the most important parameters involved in this controlled migration, believed to be important in both wound healing and embryogenesis.

1.5.4.1. Cell movement

Cells are believed to crawl forwards and change their direction of movement using a network of cytoplasmic, contractile, actin microfilaments. These microfilaments are attached to the cytoplasmic side of focal contacts and the sites of cell-substrate contact as well as the nucleus and lie parallel to the direction of movement. Protrusions at the leading edge of the cell form new focal contacts and provide traction as the cell body is pulled forward causing focal contacts already present to be situated towards the back of the cell, whilst maintaining the same position relative to the substratum. Microfilaments in the posterior part of the cell, also attached to the nucleus, detach from focal contacts and allow the posterior half of the cell to move forward. The average life span of a filament or focal contact in a migrating cell is approximately 10 minutes. Such movements are typical in wound healing, phagocytosis and embryogenesis (Abercrombie, 1982).

The interaction of these microfilaments with other cell machinery has been studied further and will be described in the context of contact guidance in section 1.5.4.2.

1.5.4.2. Contact guidance

The history of contact guidance, reviewed by Dunn (1982), reports experiments performed in 1910 by Ross Harrison. He noted the necessity for a solid support for cells to translocate and the orientation of cells by fibres from spiders' webs. Experiments by Loeb and Fisher in 1917 described the orientation of cells migrating into plasma clots under tension. In the 1940s Weiss first used the term contact guidance to describe orientation of cells by the underlying substratum. He found that Schwann cells grown on a 26 μm diameter glass fibre orientated parallel to the long axis of the fibre and that when a suspension of cells was placed on a glass substratum ruled with parallel

grooves, the cells rapidly became bipolar and elongated along the grooves, indicating directed locomotion. In the mid 1960s Curtis and Varde described the direct influence of substratum shape on cell behaviour. This was not confirmed until the mid-70s when cells were seeded on glass cylinders in culture and cell orientation and bi-directional movement were noted. Cells undergoing contact guidance should demonstrate an equal tendency to translocate in two opposite directions provided that other behavioural influences are absent (Dunn, 1982).

Dunn and Heath (1976) detected a contact guidance effect on cylindrical glass rods and prisms using chick heart fibroblasts. They related the behaviour of cells on the rods to the curvature of the rod or the ridge angle at the top of the prism, which was detected by the cell's cytoskeleton. Bending of the cell's microfilaments to an angle of 4° or greater in order for a cell to move across the ridge of the prism caused isolated cells to alter their orientation to lie parallel to the long axis of the rod or the ridge. Confluent cells could not easily avoid moving across a steep angled ridge, however cellular outgrowths were usually inhibited from crossing a ridge wall with an angle of 32°. The work with rods demonstrated orientation of cells on rods with a maximum radius of 100 μm but that the angle of curvature of larger diameter rods was too small to have an orientating effect. This threshold radius appeared to vary with cell type. Dunn and Heath concluded that the shape of the substratum imposed mechanical restrictions on the formation of linear bundles of microfilaments in the cytoplasm which are involved in cell locomotion. In 1978, Dunn and Ebendal used hydrated collagen gels as contact guidance scaffolds for heart fibroblasts or nerve axons. They discovered that air-dried gels, which were much flatter than the hydrated gels, were less effective at guiding cells and they concluded that contact guidance occurred as a result of the shape of the substratum. Chiquet *et al.* (1981) described the alignment, *in vitro*, of myoblasts along oriented fibrils or streaks of purified fibronectin.

There then followed a large amount of research focusing on grooved substrata as contact guidance scaffolds. Whereas cylindrical substrata offer a uniform guidance environment with only one altered property, curvature, grooved substrata had a number of variables including spacing of grooves and groove width, depth and altered adhesiveness of the etched surfaces (Dunn, 1982).

Clark *et al.* (1992) demonstrated alignment of BHK cells in 24 μm wide tracks on fused quartz but not on 4 μm tracks. Epithelial canine kidney cells aligned on 4-50 μm wide tracks, however cells in 2 μm wide tracks had cellular protrusions crossing other tracks. They concluded in narrow grooves cells orientated in the direction of the groove but a wide groove allowed directional deviation.

The importance of orientation of cytoskeletal elements was described by Oakley and Brunette (1993) using human gingival cells on grooved titanium surfaces or control smooth surfaces. They noted 20 minutes after cell plating that cell microtubules aligned parallel to the direction of the grooves and that this occurred before cellular orientation as a whole. After 40-60 minutes actin microfilaments were seen to have aligned, with those closest to the wall-ridge edge aligning first. Aligned focal contacts were also seen after 3 hours. Meyle *et al.* (1994) noted aligned focal contacts of human gingival fibroblasts on grooved surfaces and Wojciak-Stothard *et al.* (1995a) noted aggregation of actin microfilaments in BHK fibroblasts on a grooved surface 5 minutes after plating out. Wojciak *et al.* (1995) also reported that multiple grooved substrata could be used to facilitate the healing of rat flexor tendons *in vitro* and that murine macrophages (Wojciak-Stothard *et al.*, 1995b) showed significantly increased speed of movement and persistence of movement as well as increased F-actin content in the cells when cultured on a micropatterned surface.

Chou *et al.* (1995) noticed elongated and orientated human gingival fibroblasts on titanium coated grooved surfaces. They also found the amount of fibronectin mRNA / cell was increased on grooved surfaces compared to smooth and that the half-life of the mRNA and thus the stability was also increased. Twice as much fibronectin was found to be assembled into the matrix when cells were orientated on grooved substrata.

Contact guidance appears to rely on gross geometrical configuration to control cellular movements and activities but there has been no evidence reported of ultramicroscopic dimensions (<0.1 μm) influencing the directional behaviour of cells (Dunn, 1982).

1.6. Recombinant cell adhesion substrates

As described in section 1.3, fibronectin is a large glycoprotein with cell adhesion functions. To extract and purify large quantities of fibronectin from human plasma may be an expensive and laborious task requiring large scale gelatin affinity chromatography as well as posing a potential virus risk if the final fibronectin product was for non-autologous use. The manufacture of recombinant fibronectin could reduce both processing costs and viral threat. Unfortunately since the molecule is a large, multi-domain structure research has concentrated on recombinant cell-binding domains rather than recreating the entire molecule. Schwarzbauer *et al.* (1987) used retroviral based vectors to generate fibronectin from NIH 3T3 cells. The formed peptides lacked their N termini but were secreted by the cell, and formed disulphide bonds with themselves and with endogenous fibronectin. Kimizuka *et al.* (1991) expressed recombinant type III homology repeats in *E.coli* and found that when coupled with a number of these repeat sequences, cell adhesion activity was as good as native fibronectin.

Cappello and Crissman (1990) and Anderson *et al.* (1994) describe a combination of silk fibroin and fibronectin cell attachment domains, again expressed in *E.coli*. This new product combines the physical and chemical stability of silk fibroin but with the cell attachment properties of fibronectin. It is named Silk-like protein F (SLP-F) or Pronectin-F (Protein Polymer Technologies Inc, San Diego) and can be used to coat tissue culture dishes or to make films, both with cell attachment properties. Pronectin-F is a polymer with molecular weight 72 kDa and contains 13 RGD sequences with structural regions similar to the crystalline regions of silk.

Varani *et al.* (1993) noted that at low concentrations of Pronectin-F cells would attach to the substrate and spread. When used in combination with another substrate, such as poly-L-lysine, lower concentrations of Pronectin-F were required to achieve cell spreading. This would reduce the amount and thus the cost of the recombinant protein for large scale use.

Other research has centred around the addition of synthetically made cell adhesion peptides to polymer networks to enhance their cell adhesion properties. This again

reduces the viral risk and cost of purified human fibronectin (Barrera *et al.*, 1993; Drumheller and Hubbell, 1994).

1.7. Protein precipitation

Precipitation is said to have occurred when a previously soluble protein becomes insoluble following the addition of a reagent to the protein solution. The choice of the precipitant used will depend on whether just one protein is being extracted from a solution or whether proteins with differing sizes or solubilities are being fractionated. Changes in pH, temperature, addition of salts, organic solvents and non-ionic polymers have all been used to precipitate proteins from solution and their method of precipitation in each case will be discussed briefly. Isoelectric precipitation is most relevant to this thesis and so examples of proteins precipitated in this manner will be given.

1.7.1. Precipitation by salt addition

Neutral or slightly acidic salts, such as NaCl, Na_2SO_4, $NH_4(SO_4)_2$, have all been used to precipitate or fractionate proteins. At low concentrations salts may increase protein solubility but at high salt concentrations a process termed 'salting out' occurs. KCl, $CaCl_2$ and NaCl have all been used to solubilise and precipitate certain fibrous proteins, including myosins, collagens, fibrinogens and keratins (Englard and Seifter, 1990). Salting out occurs because the increased ionic strength of the solution pulls water molecules away from hydrophobic regions of the protein leading to increased interaction between hydrophobic patches on the protein surface and effective neutralisation of surface charges. Multivalent anions such as sulphate, phosphate and citrate are preferred in combination with monovalent cations, which do not complex with the protein. The Hofmeister series orders anions depending on their precipitating ability with citrate being the most efficient precipitant and also having the least destabilising effect.

$$citrate > PO_4^{3-} > SO_4^{2-} > acetate > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$$

The order of preference for choice of cations is $NH_4^+ > K^+ > Na^+$ although cost, the density of the solution compared to the protein and the effect on protein stability should also be considered (Scopes, 1994).

Ammonium sulphate is frequently the salt precipitant of choice because it is cheap enough to be used on a large scale, it protects proteins from denaturation in solution and limits bacterial growth, as well as a having a low heat of solution which reduces the chance of protein denaturation when the salt is added (Englard and Seifter, 1990).

1.7.2. Isoelectric protein precipitation

Isoelectric precipitation occurs when the pH of a protein solution is altered by the addition of extra acid or base causing the overall charge of the protein molecule to become zero. The pH at which the protein has no overall charge is termed the isoelectric point and is usually the pH at which the protein precipitates. When the pH is lowered below the protein's isoelectric point the molecules become protonated and repel, whereas when the pH is raised the molecules lose protons, take on an overall negative charge and repel. At the isoelectric point there is no repulsion because there is no overall charge and the particles tend to aggregate. Isoelectric precipitations tend to depend upon the components present and the behaviour of a protein in a mixture may be different from its behaviour in a pure solution (Scopes, 1994).

Gentle heat and isoelectric precipitation (pH 4.2) have been described to fractionate whey proteins (Bramaud *et al.*, 1997) whilst the protein from sunflower seed has been extracted around its isoelectric point, pH 3.6-4.0 (Taha *et al.*, 1981; Rapheal *et al.*, 1995; Rapheal and Rohani, 1996). Soybean protein has been extensively investigated and precipitated around its isoelectric point between pH 4.0 and 4.8 (Lillford and Wright, 1981; Virkar *et al.*, 1982).

The examples given above all precipitated in acid conditions, however this is not always the case with some proteins being stable in acidic solutions and precipitating under alkaline conditions. Mineral acids are frequently used for protein precipitation because they are cheap and hydrochloric, sulphuric and phosphoric acids are accepted for human consumption and therefore do not necessarily have to be removed from the final product (Bell *et al.*, 1983).

1.7.3. Precipitation using organic solvents

Solvents such as methanol, ethanol, butanol and acetone have been used to precipitate proteins. All the solvents have both hydrophilic and hydrophobic groups which results in the solvent interacting with the hydrophobic regions of the proteins and competing with the water to interact with the hydrophilic regions. The solvating power of the water is decreased as the concentration of solvent increases, causing molecules to aggregate. Aggregation is increased at the isoelectric point and large proteins require lower concentrations of solvent to precipitate out. As described in section 1.2. ethanol fractionation is frequently used in the plasma fractionation industry. However there are disadvantages associated with the use of solvents including a tendency to denature proteins at temperatures above 0°C and the need to design the plant for use with flammable solvents (Englard and Seifter, 1990; Scopes, 1994)

1.7.4. Precipitation using non-ionic polymers

The action of non-ionic precipitating agents such as PEG has been described previously in section 1.2. along with their use in the plasma fractionation industry. The action of these polymers as precipitants can be enhanced by alteration of pH towards the isoelectric point of the protein or with the addition of divalent metal ions to complex the protein (Scopes, 1994). Ingham (1990) describes the precipitation of fibronectin with 11 % PEG from a solution of phosphate-buffered saline. However a concentration of only 3 % PEG was needed when a solution of fibronectin and gelatin was being precipitated. Gelatin was found not to precipitate alone under these conditions thus associations between proteins in solution are important during precipitation.

1.7.5. Other less frequently used forms of precipitation

Since not all proteins have the same pH and temperature optima, manipulation of environmental conditions can selectively denature some proteins whilst leaving others intact. Altering temperature, pH and the presence of organic solvents, frequently in combination with each other, can result in the loss of the protein's tertiary structure and the formation of free polypeptide chains. These will interact randomly and cause precipitation of the denatured protein. This happens at extremes of pH when molecules gain or lose charges which were involved in the stabilisation of the structure (Scopes, 1994).

Ionic polyelectrolytes, including alginate and carboxymethylcellulose, have also been used in the food industry to precipitate proteins by disrupting the molecular electrostatic interactions. On the addition of carboxymethylcellulose, precipitation has been found to occur below the protein's isoelectric point (Bell *et al.*, 1983).

It can be seen that there are a number of ways of precipitating proteins but the method selected depends upon the stability of the protein, its final use and the need to remove contaminating precipitants, the economics of the project, design of plant and the separation problem. Precipitation is an important feature of fibre wet spinning which will be discussed next.

1.8. Fibre wet spinning

1.8.1. Wet spinning

Fibres are formed when a high concentration polymer solution, termed dope, is extruded through a hole as a liquid threadline into a non-solvent. This non-solvent precipitates the polymer and extracts the solvent resulting in 'coagulation'. The hole through which the polymer is extruded is part of the spinneret. Large scale, commercial spinning processes for the manufacture of viscose rayon or acrylic fibres use large spinnerets with many thousands of holes. Fibre formation occurs due to the shearing effect on the polymer, aligning molecules, as it is extruded through the spinneret combined with coagulation. Fibre formation is relatively slow in a wet spinning process due to the time required for the fibre to solidify and fibre collection is usually of the order 10 -150 m/min. Fibres are traditionally washed extensively to remove the often aggressive coagulating agent, drawn to increase their internal orientation and wound onto collecting rollers and dried.

1.8.2. Fibre orientation

Fibres have a high molecular weight and are made up of a number of smaller monomers joined by either condensation or polymerisation reactions. This high molecular weight polymer must have a longitudinal structure in order to be fibrous. Following extrusion fibres are stretched to further align the individual molecules and orient them parallel to the long axis of the fibre. With increasing orientation fibres become more crystalline as the molecules unfold and their arrangement in the fibre becomes more orderly. The

molecules can slide past each other during the drawing process, if they are not cross-linked, resulting in improved packing and the formation of many hydrogen bonds, increasing the overall strength. Properties of highly oriented fibres, such as nylon, are high strength, low elongation at break, brittle, high lustre and low moisture absorption due to the inability of water molecules to penetrate between the tightly packed molecules. Many natural and regenerated proteins, such as wool and casein, are poorly orientated fibres. This is because the individual molecules are essentially globular in their natural state and have to be unfolded in the process of fibre formation. The properties of such fibres are low strength, pliability, low lustre and high moisture absorption (Moncrieff, 1969).

The basic requirements for fibre formation are:
- linear molecules with relatively high but not extreme molecular weight
- high degree of polarity for good intermolecular cohesion
- high degree of linear symmetry and absence of bulky side chains so that molecules are tightly packed and good orientation is achieved. Wool fibres have bulky side chains and do not crystallise well whilst silk fibres, with their small side chains, are well orientated (Huang and Rha, 1974). Increasing shear aids the orientation of molecules, although shear through the spinneret is only a preliminary feature of fibre orientation with alignment of the fibre after precipitation by stretching and drawing also playing an important role (Huang and Rha, 1974).

1.8.3. Protein wet spinning

Interest in spinning protein fibres began in the 1890s, but it was not until 1935 when commercially acceptable casein fibres with wool-like properties were spun (Moncrieff, 1969). Protein fibres are made up of high molecular weight polypeptide chains and the individual composition of amino acids in each chain confers properties to the final fibre. As described above fibres made from proteins usually have poor internal orientation and this is seen from data for commercially produced fibres made during 1930s-1960s, where they were favoured for use in wool mixes (Table 1.4). Soybean protein fibres were found to have the most wool-like properties.

Protein	Fibre name	spun from - to	Tenacity (g/denier)	Elongation at break (%)
Casein	Lanital, Aralac, Fibrolane Merinova	caustic soda to sulphuric acid, formaldehyde and glucose mix	dry: 0.8-1.1 wet: 0.6	15-60
Zein	Vicara	caustic soda to acid / formaldehyde	dry: 1.2 wet: 0.75	dry: 32 wet: 35
Groundnut	Ardil	dilute caustic soda to sulphuric acid/ sodium sulphate, treated with formaldehyde	dry: 0.8	50
Soybean	Silkool	caustic soda to acid	dry:0.8 wet: 0.25	50

Table 1.4. *Summary of spinning conditions and properties of protein fibres once spun commercially in the textile industry. Fibres made by different companies were often given different names, a selection of which are given above. Tenacity is a measure of strength with respect to fibre fineness. For a highly orientated fibre, such as nylon, the tenacity has been measured as 7 g/denier (Moncrieff, 1969).*

Some proteins exist in a naturally fibrous state, e.g: collagen, actin, myosin, keratin and silk, whilst the natural state for other proteins is a more globular, tightly packed conformation. To spin fibres from these globular proteins it is essential to first convert them to an extended conformation. This can be done by heat or solvents which disrupt bonds maintaining the tightly packed structure and produce flexible polypeptide chains, without degrading the protein structure. Urea, guanidine salts, formaldehyde, sodium salicylate, various detergents and some inorganic salts such as lithium iodide have been used to produce suitable spinning solutions. As the molecules open out into their extended conformation and interact, there is an increase in solution viscosity. This increase in viscosity aids formation of fibres and solutions of dissolved protein are often left to 'ripen' to improve their spinning properties. Proteins which have been converted from compact to fibrous forms by heat or various solvents include casein, lactoglobulin, zein, haemoglobin, soybean, peanut, ovalbumin, edestin, tobacco seed globulin, pumpkin-seed globulin, albumin, globulins and mixed proteins of horse serum (Senti *et al.*, 1945).

In general, a concentrated, viscous solution of protein, containing 15-30 % protein, is spun into a bath of aqueous acids, inorganic salts and frequently a heavy metal ion. These fibres are then stretched and hardened with formaldehyde and acetic anhydride, with the tightly packed chains being stabilised by dissociable salt linkages and hydrogen bonds (Lungren, 1949). Many hydrogen bonds can form along the length of the molecules giving overall strength. Some proteins such as albumin and feather keratin form stiff gels at the high concentrations required for extrusion. Dissolution in high concentrations of alkali can have a hydrolytic effect on the peptide chain but using ionic detergents to unravel the polypeptide chains was found to assist in the solubilisation of the more awkward proteins (Lundgren, 1949). In 1945, Asteury noted that X-ray diffraction patterns of globular proteins dissolved in urea, as preparation for spinning from solution, were similar to those for fibrous, extended molecules, such as fibrous keratin.

Traill (1945) describes the development of a wet spinning technique for the production of ground nut (peanut) fibres. Originally solutions of 30 % (w/v) protein were dissolved in 5.8 M urea. These were precipitated into 1 % sulphuric acid with 15 % sodium sulphate and hardened with formaldehyde to give filaments with wool-like characteristics. He found that the highly soluble urea sulphate, formed in the coagulating bath, was difficult to recover and so alkali, typically sodium hydroxide, became the solvent of choice for wet spinning protein. He found that adding sodium chloride or other salts to the coagulating bath reduced instances of the fibre sticking together whilst the acid concentration was kept below 2 % to reduce the possibility of acid hydrolysis of the protein chains. Sulphate ions were found to reduce hardening by the formaldehyde.

The commercial production of protein fibres for textile uses was halted at the end of the 1960s mainly due to the fibre's poor wet strength. Earlier, in 1954, Boyer adapted spinning processes which had traditionally been used to manufacture textiles to produce food materials with enhanced appeal to customers (Huang and Rha, 1974). Fibres have since been made from a variety of animal and vegetable proteins including: porcine lung and stomach, bovine plasma, (Young and Lawrie, 1974, 1975a,b) porcine plasma, (Swingler and Lawrie, 1977) zein, (Balmaceda and Rha, 1974) and fish proteins (Mackie and Thomson, 1982). Soy protein fibres have been extensively researched for the production of meat-free textured products and there are a number of patents

describing methods for their manufacture (Tombs, 1975; Segeren and Boskamp, 1977) as well as patents for vegetable protein fibres, (Sternber and Kim, 1976) protein fibres with additives such as polysaccharides (Sawada *et al.*, 1974) and unsaturated fatty acids (Yano *et al.*, 1974), fish proteins (Askman *et al.*, 1982) and casein plus a heat-settable protein (Visser *et al.*, 1978).

There has also been interest in spinning proteins (and polysaccharides) in the production of biomaterials. Cavallaro *et al.* (1994) describe the spinning of collagen fibres from a dilute acetic acid solution into a neutral polyethylene glycol coagulation bath, whilst chitin and chitosan are two polysaccharides which have been wet spun (East *et al.*, 1989) and have potential use in wound dressings. Ferry (1948) also describes the production of denatured fibrin gels using similar protein denaturation / aggregation techniques to those required for wet spinning.

1.9. Fibronectin mats as contact guidance materials

The production of fibronectin tissue repair scaffolds originated from research by Brown and co-workers in the Tissue Repair Unit, University College London (Ejim *et al.,* 1993; Brown *et al.*, 1994) and it is this work, discussed below, which provides the foundation for the problem tackled in this thesis

Purified fibronectin was aggregated and self-associated under conditions of solution concentration and directional shear to produce mats comprising orientated fine protein fibrils (Ejim *et al.*, 1993). The mats were produced from affinity chromatography purified fibronectin solutions from a glycine supernatant fraction of the plasma fractionation process (BioProducts Laboratory, Elstree, UK) containing 0.3 M NaCl, 2 M urea and 0.05 M tris-HCl. After washing and freeze drying, the mats had dimensions 1 x 2 cm, were several millimetres thick, and formed a network with interconnecting pores (size < 10μm - 210 μm) between the orientated fibrils (Ejim *et al.*, 1993). Fibronectin incorporation into mats was found to be maximal in the presence of 0.1-0.3 M NaCl and decreased in the absence of urea (Brown *et al.*, 1994) suggesting that the formation of the extended form of the fibronectin molecule was critical to self-aggregation.

Heparin, known to form precipitatable complexes with fibronectin in the cold (section 1.4.3), could be incorporated into the mats either during manufacture or after formation of the mat but before drying. The heparin containing mats had a similar fibrillar appearance to the plain fibronectin mats but were less stable in solution, dissolving within 24 hours in a phosphate-buffered saline (PBS) solution (Ejim *et al.*, 1993) Cross-linking these heparin mats with carboiimide gave them stability for 14 days, equivalent to plain mats, in a cell free medium (Ejim *et al.*, 1993). The mats were found to be hygroscopic and swelled on rehydration to give final water contents of 6x and 4x their original dry weight for plain and heparin containing mats respectively (Brown *et al.*, 1994). Incubation in PBS at 37°C for two days resulted in the loss of 50 % of total protein for the plain mat and 15 % for the cross-linked heparin containing mat (Brown *et al.*, 1994). Both types of mat bound basic Fibroblast growth factor (b-FGF), which is believed to be an important factor in angiogenesis and thus wound repair. Cross-linked heparin mats released this factor more rapidly than the plain mats but both still retained some factor after 48 hours, demonstrating the potential for using these mats as a depot for growth factors. Fibroblasts did not appear to increase degradation of the mats under culture conditions (Brown *et al.*, 1994).

Fine fibronectin strands, length 1-5 mm and diameter 1-10 μm, could be drawn from both solutions of purified fibronectin and fibronectin - heparin solutions. Each strand was found to be composed of a number of tightly bound fibrils. Assessment of the interaction of rat tail tendon and human skin fibroblasts with these strands and the fibronectin mats showed that cells attached to both mats and strands. Cells were seen to be orientated by the strands and took on a polarised morphology which was evident in cells several cell diameters away from the strand. Fibroblasts were seen both on top and within the mat (Ejim *et al.*, 1993).

Wojciak-Stothard *et al.*(1997) describe promotion of cell spreading and alignment of various cell types along fine fibronectin strands as well as alignment of actin filaments and focal contacts in rat tendon, baby hamster kidney fibroblasts and macrophages. Polymerization of F-actin was increased as well as the speed and persistence of cell movement. They concluded that the 3-D fibronectin substrate was more effective than 2-D fibronectin covered glass or plain glass for activating cells.

Fibronectin mats have also shown successful results when combined with cells. Prajapati *et al.* (1996) describe a potential application of mats, as carriers for keratinocytes for use in epidermal grafting, when treating the inherited skin blistering condition epidermolysis bullosa. After incubation for seven days, the mats were invaded by keratinocytes and acted as a substrate for their adhesion with the cells maintaining their migratory phenotype, essential for tissue repair. Implantation of fibronectin mats into full-thickness porcine wounds showed regeneration of new tissue parallel to the orientation of the implanted mats (Brown *et al.,* 1998a). Blood vessels were seen growing into the wound from the deep tissue towards the surface, perpendicular to fibronectin directional cues, and must therefore be associated with growth factor gradients (Brown et al., 1998b)

Both the fibronectin mats potential clinical applications, as a depot for growth factors and as a scaffold for orientated tissue regeneration, were tested in *in vivo* nerve regeneration experiments in rats. Mats were used to bridge 1 cm gaps in rat sciatic nerves and showed regeneration comparable with a homologous nerve graft and better than a homologous muscle graft. A high level of macrophages were seen initially associated with the mat but these reduced 30 days after implantation. The mat appeared to have degenerated after 14 days but there was no evidence of excess scar tissue (Whitworth *et al.*, 1995a). Axonal regeneration was also noted in diabetic rats (where levels of nerve growth factor (NGF) are believed to be diminished) (Whitworth *et al.*, 1995b) and non-diabetic rats (Whitworth *et al.*, 1996) when fibronectin mats impregnated with NGF were implanted into rat sciatic nerves.

Fibronectin mats have been stabilised using micromolar concentrations of copper ions, which increase the length of time the scaffold remains in culture. The concentrations of copper required are non-toxic to even highly sensitive Schwann cells (Ahmed *et al.*, 1998a).

Thus it appears that both fibronectin mats and strands can be used as *in vivo* and *in vitro* cell orientation scaffolds by virtue of their topography and cell adhesion properties and fibronectin mats may find use as growth factor release depots.

1.10. Project aims

Initially the main aims of this project were:

- to investigate whether fibronectin mat-making is a scaleable process and whether a solution from an alternative fractionation centre (PFC, Edinburgh) could be used to produce such materials.

- To identify a method of reproducibly producing a fibronectin material which has good cell adhesion properties and serves as a template to orientate cells.

- To test materials manufactured for their suitability for use in skin replacement therapy.

- To identify a manufacturing process which is scaleable and could be operated under Good Manufacturing Practice conditions.

2.0. Materials and Methods

2.1. Precipitate preparation

Frozen cryoprecipitate was thawed overnight, at 4°C, shredded and dissolved in 5 times w/v buffer A, (0.01 M Na_2HPO_4, 0.01 M $NaH_2PO_4.2H_2O$, 0.15 M NaCl, pH 7.0), at 37°C. Following cooling to room temperature, 4 % (w/v) polyethylene glycol 4000 (PEG 4000, BDH, Poole, UK), was added and left for one hour at room temperature to precipitate any fibrinogen present. The precipitate was then removed by centrifuging at 4500 r.p.m. for 15 minutes to form a protein pellet. The concentration of PEG 4000 in the supernatant was increased to 10 % (w/v) and left overnight to precipitate out the remaining proteins. Pellets of protein were again formed by centrifuging at 4500 r.p.m. for 15 minutes. These pellets were either stored frozen or dissolved into buffer B, (buffer A + 25 mM trisodium citrate). Solutions were made so that the final fibronectin concentration was approximately 3 mg/mL. The solution was clarified by filtration through a 0.2-0.8 μm filter, followed by sterile filtration (0.2 μm) into 2 litre bottles. Large bottles of solution were stored frozen at -20 °C, until needed.

Solutions could then be defrosted in a water bath, 30-37°C, divided into 20 mL aliquots and refrozen as required. The number of freeze-thaw cycles was kept to a minimum although at least 5 cycles could be performed without alteration to the cable making process.

2.2. Methods of solution characterisation

The protein solution formed from the cryoprecipitate did not contain pure fibronectin, so a number of different assays were used to investigate the solution's composition. Standard curves and assay errors are given in Appendix 10.1.1. All assays were performed in duplicate.

2.2.1. Protein assay

The Bradford protein assay, a dye-binding assay, was used to measure the total protein in solution. The assay is based on a colour change and shift in absorbence maximum when the dye, Coomassie Brilliant Blue G-250 in an acidic solution, binds to protein.

10 μL of standard / sample, concentrations 0.2-1.0 mg/mL, were added to 250 μL dye reagent concentrate (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) pre-diluted 1 part reagent with 4 parts deionized water. The assays were carried out in duplicate in a 96 well plate (Sarsedt, Leicester, UK) and the resulting absorbence measured against a reagent blank at 570 nm, using a Dynatech 7000 plate reader, (Dynatech Laboratories, Billingshurst, UK). Protein standards used were a mixture of albumin and globulins (Sigma, Poole, UK)

2.2.2. Fibronectin ELISA

A two-antibody, non-competitive ELISA was developed to quantify the amount of fibronectin in the samples. ELISA development is described in section 3.2.1 and the procedure outlined in Figure 2.1.

Affinity chromatography purified fibronectin was used as the standard for the ELISA. Purification of fibronectin is described in section 2.3.1.1. The fibronectin concentration was calculated from the solution's absorbence at 280 nm using the extinction coefficient for the protein as shown below:

$$[Fn]mg/mL = \frac{A280}{1.26}$$ (Iuliano *et al.*, 1993)

This stock solution was then diluted in 0.01 M phosphate-buffered saline, pH 7.4 (PBS) to give concentrations between 0.03 and 2 μg/mL. 200 μL of each sample / standard was added to duplicate wells of a Maxisorp 96 well immunoassay plate (Life Technologies Ltd, Paisley, Scotland) and allowed to incubate overnight at 4°C. Incubation was terminated by the addition of 150 μL 0.01 M PBS plus 0.02 % (v/v) polyoxyethylenesorbitan monolaurate, (Tween 20), (Sigma) followed by rinsing twice with deionized water. Any remaining binding sites on the plate were blocked with 100 μL blocking buffer, (150 mM NaCl, 100 mM tris, pH 9.0, 1 % (w/v) bovine serum

albumin) and allowed to incubate for 30 minutes at room temperature. Incubation was again terminated with PBS-Tween and the plate washed, as before.

The concentrations of antibody added were determined by an optimisation experiment. The primary antibody, rabbit anti-human fibronectin (Sigma) was diluted 10 000 fold in PBS plus 1 % (w/v) bovine serum albumin and 100 µL was incubated in each well for 1 hour at room temperature. Incubation was terminated by washing the plate as before. Following washing, 100 µL of the secondary antibody conjugate, goat anti-rabbit, alkaline phosphatase linked IgG (Sigma) diluted 4 000 fold was added and allowed to incubate for one hour at room temperature. The plate was washed, as before. The enzyme substrate was 100 µL disodium paranitrophenyl phosphate (pNpp) in diethanolamine buffer (Sigma). The substrate solution was made up as follows: pNpp 2 mg/mL, diethanolamine 10 % (v/v), pH 9.8, 0.5 mM $MgCl_2$. Plates were incubated at 30°C for between 30 minutes and 2 hours until the most concentrated samples gave an absorbence of approximately 1.0 at wavelength 410 / 490 nm.

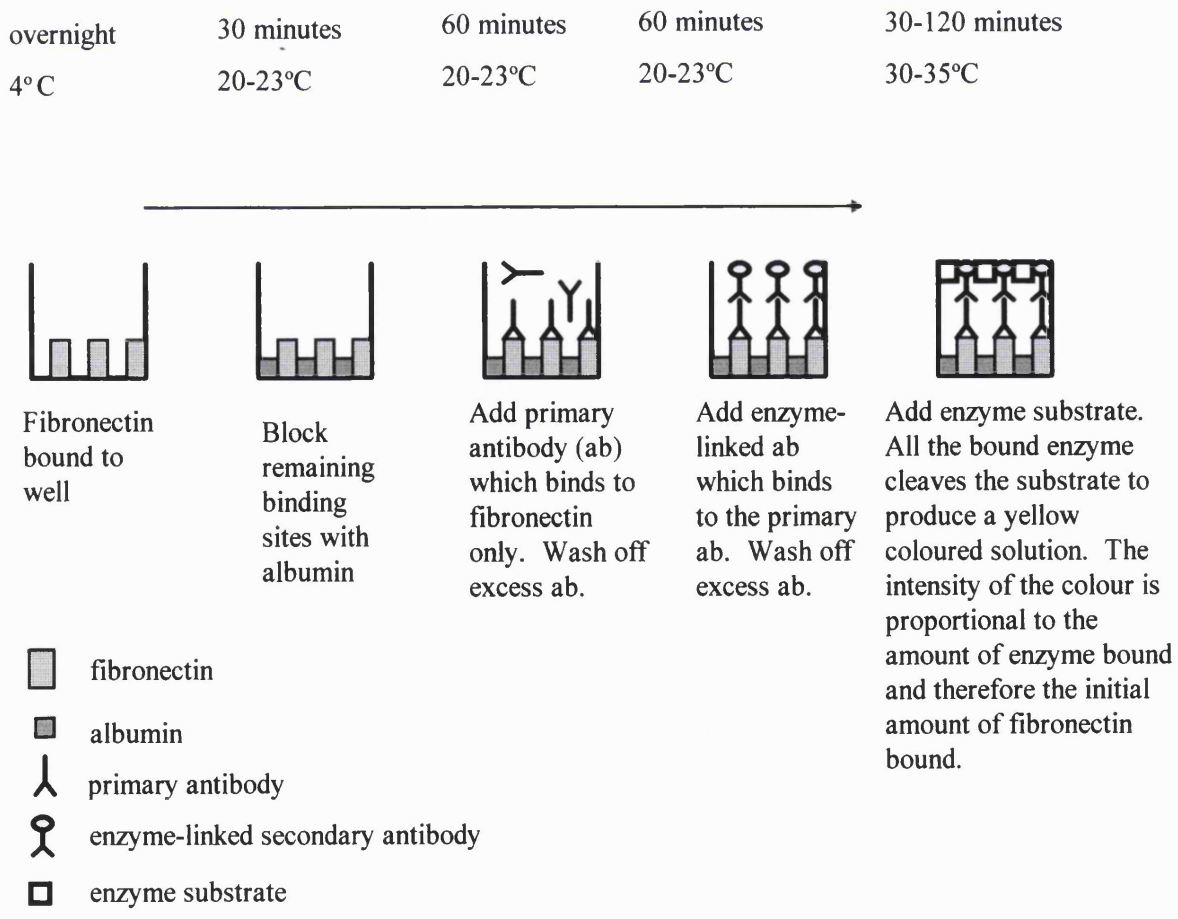


Figure 2.1. *Principle used for the two antibody fibronectin ELISA.*

2.2.3. Fibrinogen assay

Sample fibrinogen concentration was measured by heat precipitation of the protein.

Fibrinogen concentrations were measured against fibrinogen standards (Protein Fractionation Centre, Edinburgh). Standards and samples were prepared in the concentration range 0.3-5.0 mg/mL using PBS as the dilutent. 125 μL sample was then mixed with 125 μL buffer (20 mM tris, 40 mM tri-sodium citrate dihydrate, pH 7.5) and heated for 15 minutes at 56°C. Absorbence was measured against a buffer blank at 410 nm.

2.2.4. Polyethylene glycol assay

Polyethylene glycol 4000 (BDH) was diluted to give a concentration range 0.6-10 μg/mL. Samples were diluted into this concentration range using PBS, pH 7.4. Samples were then diluted 10 fold with 7 % (w/v) perchloric acid (BDH) and centrifuged for 10 minutes, to precipitate out proteins present. 1 mL of supernatant was added to 0.25 mL of acidified barium chloride (5 %(w/v) $BaCl_2$ in 1 M HCl) and left for 10 minutes at room temperature. 0.125 mL of 0.05 M iodine was then added and the solutions left at room temperature for 15 minutes. The absorbence of the solution was measured against a reagent blank at 535 nm.

2.2.5. Albumin assay

Measurement of albumin used an anionic dye, bromocresol green in a succinate buffer, pH 4.2. This dye binds tightly to albumin, where present, and causes an increase in light absorption over unbound dye at 628 nm (Kaplan and Szabo, 1983).

5.0 mL of working bromocresol green solution (BDH) was added to 25 μL of bovine serum albumin, concentration range 0.3 - 20 mg/mL, or to samples diluted with PBS into this range. Solutions were mixed by gentle inversion and allowed to stand for 10 minutes at room temperature before the absorbence was read at 628 nm against a reagent blank.

2.2.6. Viscometry

Viscosity measurements were made to examine the flow properties of the fibronectin

solutions. The Contraves Rheomat 115 (Contraves Industrial Products Ltd., Ruislip, Middlesex) linked to a Haake K15 glycol bath (Gebruder Haake GmbH, Karlsruhe, Germany) was used for temperature controlled viscosity measurements.

The Contraves Rheomat is a rotational viscometer which operates with a variety of coaxial measuring systems. Those used in this study were concentric cylinders (MS-0) and cup and bob sets 108, 125 and 114. Dimensions of these systems are given in Table 2.1.

	Volume of sample (mL)	Range of shear rates (s^{-1})	Radius of cup R_c (cm)	Radius of bob R_b (cm)	R_c / R_b
MS - 0	18.00	24.3 - 3680	outer: 2.40 inner: 2.22	outer: 2.35 inner: 2.27	outer: 1.02 inner: 1.02
DIN 125	17.50	6.6 - 1007	1.35	1.25	1.08
DIN 114	3.50	6.6 - 1007	0.80	0.70	1.14
DIN 108	0.75	6.6 - 1007	0.45	0.40	1.12

Table 2.1. *Summary of measuring system dimensions for Contraves Rheomat 115. The calculation of shear speed is a function of the bob's actual rotational speed. The range of speed steps used is fixed by the use of plug-in modules. In this study the 7,7 module was used. No correction to the mean shear rate has been made where $R_c/R_b >$ 1.05.*

The calculation of viscosity in this system is based upon the measurement of the torque exerted on the bob whilst the bob rotates in the fluid. This torque measurement can then be converted into a value for shear stress using pre-determined constants for the system, equation 2.1.

$$\tau = \frac{M}{2\pi r^2 h} \qquad \text{(equation 2.1)}$$

Plots of shear rate versus shear stress were made for each sample. The Newtonian characteristics of the fluid were investigated by fitting curves to the data according to the power law equation, equation 2.2.

$$\tau = K\gamma^n \qquad \text{(equation 2.2)}$$

For Newtonian fluids (n=1), at a constant pressure and temperature, the viscosity of the solution could be calculated from the gradient of the line fitted to a plot of shear rate versus shear stress, equation 2.3.

$$\mu = \frac{\gamma}{\tau} \qquad \text{(equation 2.3)}$$

For non-Newtonian fluids, the apparent viscosity of the solution (μ_a) was calculated for a given shear rate.

Measurements were made over a range of both increasing and decreasing shear rates with the sample being sheared for 15 seconds at each shear rate. This data was then examined for deviation from laminar flow, pseudoplastic behaviour and reproducibility. Measurement of power law parameters and errors in their measurement are discussed in Appendix 2. Silicone oil, glycerol and water were used as standard viscosity solutions.

2.2.7. Gel electrophoresis

Protein samples were analysed by denaturing, reducing gel electrophoresis to indicate qualitatively the presence or absence of fibronectin and fibrinogen. Fibronectin consists of two 220 kDa chains linked by disulphide bonds whereas fibrinogen consists of 2 disulphide linked sets of 3 chains, size 64, 57 and 48 kDa. To achieve good separation of proteins with this wide range of molecular weights, pre-cast 8-16 % tris-glycine gradient gels were used, (R&D Systems Europe Ltd, Abingdon,UK). The gels contained approximately 2.6 % bis-acrylamide, pH 8.6 and were loaded with 20 μL sample, total protein concentration 1 mg/mL. Molecular weight standards used were wide range markers 6.5- 205 kDa (Sigma). Gels were run for 90 minutes with a constant voltage 125 V and a current of 30-40 mA/gel. Gels were stained with universal

instaview stain (BDH), a Coomasie blue based stain, for several hours. Excess stain was removed with repeated washing in distilled water and gels were dried for 24 hours between cellophane sheets using a solution of 20 % methanol, 2 % glycerol in deionized water.

Details of sample and running buffer composition and molecular weight standards are given in Appendix 1, section 10.1.2.

2.2.8. Dialysis

Buffer exchange, where required, was achieved by dialysis. Dialysis tubing (Sigma) was washed in running tap water for 3-4 hours to remove excess glycerine from storage. 20 cm lengths of tube were then soaked for 1 minute in 0.3 % (w/v) sodium sulphide to remove sulphur compounds followed by a 2 minute wash with hot water. The tubing was then acidified with 0.2 % (v/v) sulphuric acid followed by a hot water wash to remove the acid. Lengths of tubing were stored in 70 % ethanol until required and rinsed in deionized water before use.

A 20 mL sample was placed in lengths of dialysis tubing and sealed at both ends. The tubing was then placed in a 1 litre of the exchange buffer and left at 4° C for 12 hours. The buffer was replaced after 12 hours and buffer exchange allowed to continue for a further 12 hours. Exchange was enhanced by agitation using a magnetic stirrer.

2.3. Methods of material manufacture

Details described below are the optimised manufacture method. Techniques used to determine these methods are described in the appropriate results chapter. Schematic diagrams demonstrating how methods of making fibronectin materials were developed during the course of this project are shown in Figures 2.2a-d

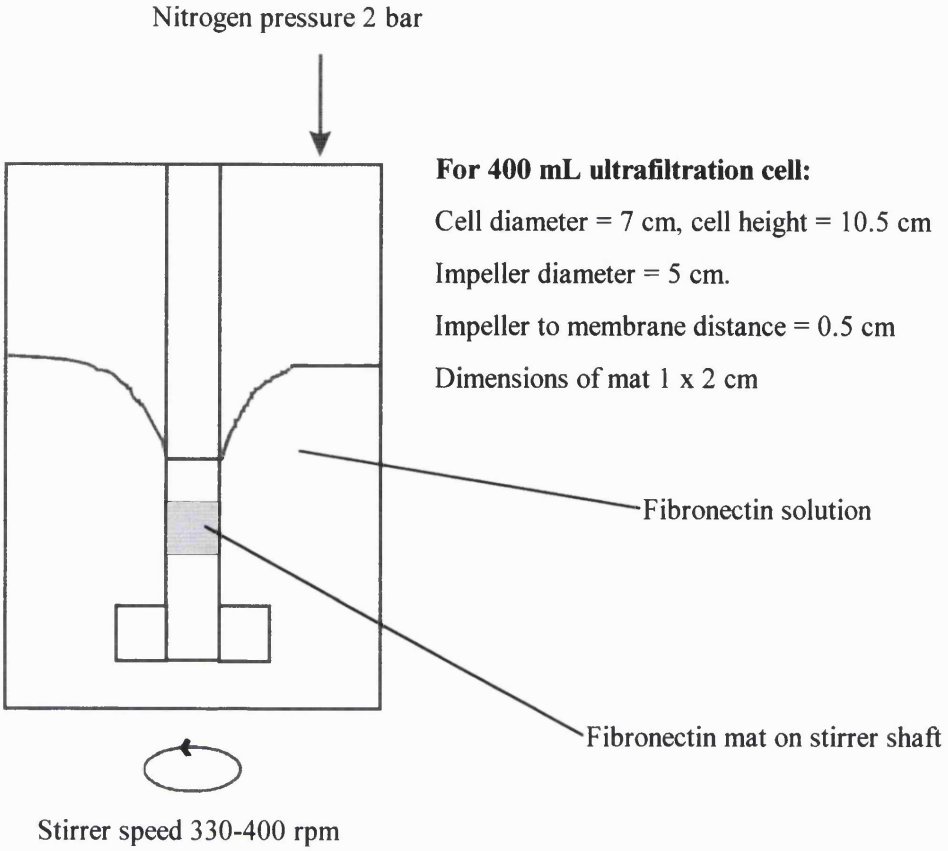


Figure 2.2a

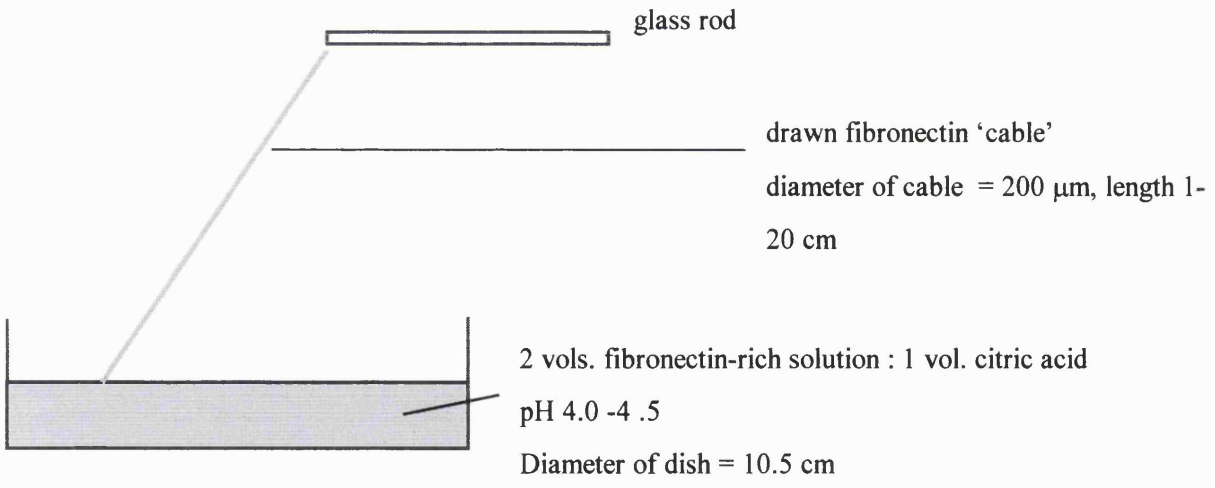


Figure 2.2b

Schematic diagrams of equipment used to manufacture fibronectin materials.

Figure 2.2a. *Fibronectin mat manufacture in stirred ultrafiltration cell.*

Figure 2.2b. *'Cables' of fibronectin are drawn upwards from a non-purified fibronectin solution.*

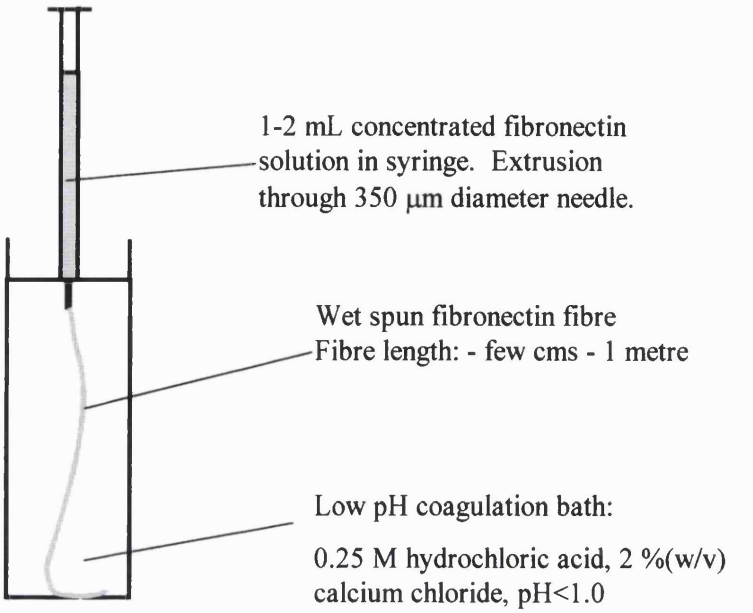


Figure 2.2c

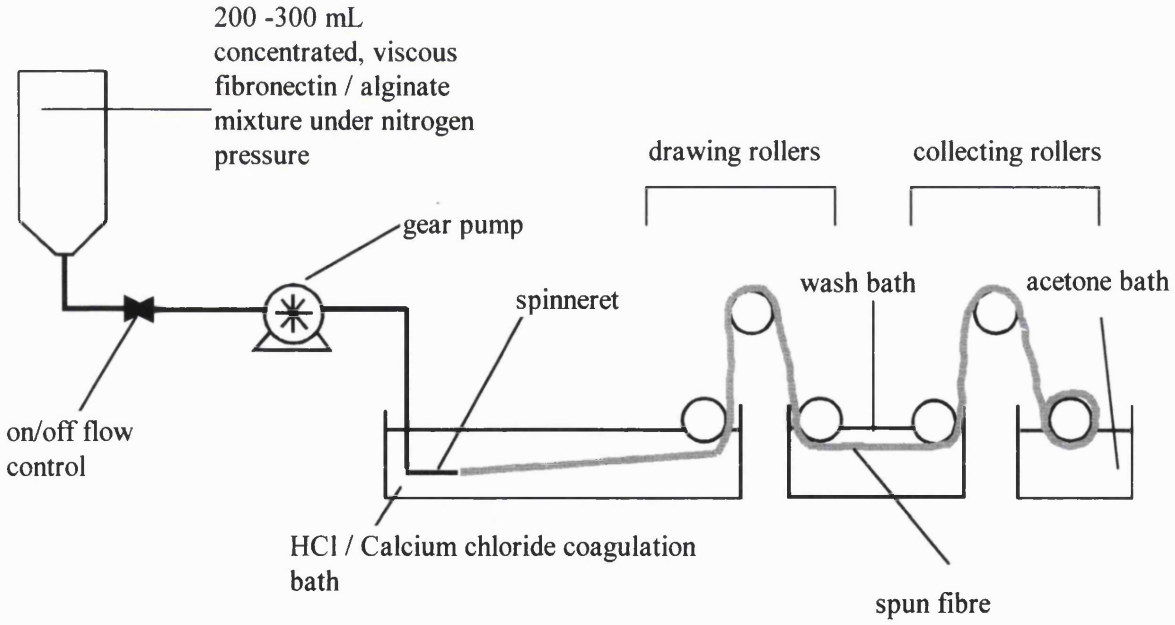


Figure 2.2d

Schematic diagrams of equipment used to manufacture fibronectin materials.

Figure 2.2c. *Small scale wet spinning. Concentrated fibronectin solutions are extruded from a syringe through a fine needle into an acidic bath to form a fine, continuous fibre.*
Figure 2.2d. *Pilot scale wet spinning. Concentrated fibronectin is mixed with alginate to enhance production of wet-spun fibres on a large scale (several metres).*

2.3.1. Fibronectin mat manufacture

2.3.1.1. Affinity chromatography

Fibronectin can be purified from plasma derivatives by passing it down a gelatin affinity chromatography column (Scott *et al.*, 1981; Morgenthaler *et al.*, 1984; Stol *et al.*, 1987; Regnault *et al.*, 1988).

Fibronectin purification was carried out using a Pharmacia Biotech XK Column, length 20 cm, internal diameter 1.6 cm, (Pharmacia Biotech. Ltd, Milton Keynes, UK) loaded with 25 mL cyanogen bromide activated gelatin immobilised on cross-linked 4 % beaded agarose (Sigma). The gelatin was initially washed with 150 mL washing buffer (50 mM tris, 0.5 M NaCl, pH 7.6) at a flowrate 1.5 - 4.0 mL/min. The sample to be purified was then loaded and the column again washed with 150 mL washing buffer before elution with 120 mL column eluting buffer (4 M urea, 50 mM tris, 0.5 M NaCl, pH 7.6). Results of flowrate alteration and column capacity estimation are given in 3.4. All affinity chromatography was carried out at 4°C.

2.3.1.2. Fibronectin mat manufacture

Fibronectin mats were made according to the method detailed by Ejim *et al.* (1993) and Brown *et al.* (1994). Affinity chromatography purified fibronectin, concentration > 2 mg/mL in 4 M urea, 50 mM tris, 0.5 M NaCl, pH 7.6 was diluted with an equal volume of buffer (50 mM tris, 0.1 M NaCl, pH 7.6) to give final concentrations fibronectin > 1 mg/mL, 2 M urea, 50 mM tris, 0.2 M NaCl.

200 mL of fibronectin solution were placed in an ultrafiltration cell (Amicon, Stonehouse, UK) containing a membrane with a 10 kDa molecular weight cut-off, PM10 (Amicon). Stirrer speed was set at 330-400 rpm so that the tip of the vortex in the unbaffled cell was at least 50 % of the height distance from the top of the cell. Temperature was kept at 4°C, filtration flowrate was 0.5-1.0 mL/min at a steady state and nitrogen pressure was 2 bar. The solution was left in the ultrafiltration cell to be sheared and concentrated for 15 minutes to 1 hour or until a mat of fine protein fibres

was seen on the stirrer shaft. The shaft and mat were then removed, washed in deionized water, frozen at -20°C overnight and freeze dried for 24 hours before the mat was removed from the shaft. Further fibronectin mats could be made from one solution if the concentration of protein remained above 0.5 - 1.0 mg/mL.

Ultrafiltration membranes were soaked in 0.1 M NaOH for several hours after use before storage in 10 % ethanol at 4°C.

2.3.2. Cable manufacture

Fibronectin-rich precipitates which could be drawn out of solution to form thin 'rods' of protein were named cables. Details of the optimisation of cable manufacture are given in section 4.4. The method used for production of material for characterisation of its physical properties is given below.

Fibronectin-rich solutions with total protein concentration 4.8 mg/mL were mixed with 0.1 M citric acid in the ratio 2:1 (v/v) protein to acid to give a final pH of 4.0-4.5. The precipitating protein fibres in the dish were gently aligned using a fine glass pipette to agitate the solution. The precipitate could then be slowly drawn upwards from the solution surface attached to the glass pipette, washed in PBS, and allowed to air dry by attaching the cable between two fixed points, e.g. the edges of a petri dish. Some cables were twisted as they were drawn by rotating the glass rod as it was drawn upwards.

2.3.3. Small scale fibre spinning

Fibres were spun from protein solutions containing greater than 70 mg/mL total protein. These solutions were made by dissolving the fibronectin / fibrinogen / PEG precipitate in 6 M urea so that a high protein concentration could be reached. Typically 1 g of freshly thawed protein precipitate would be added to 1 mL of 6 M urea and then allowed to dissolve at room temperature for several hours using the Spiramix (Jencons Scientific Ltd, Leighton Buzzard, UK) for gentle non-foaming agitation. The resulting viscous solution was then passed through a syringe-mounted glass fibre pre-filter (BDH) to remove large particulate matter.

Fibres were spun from a 1 mL syringe through a hypodermic needle (orifice diameter 350-1200 μM) into a coagulation bath containing 0.25 M HCl, 2 % $CaCl_2$, pH <1.0. To reduce the possibility of needle blockage, the main barrel of the needle was removed and the solution was extruded only through the orifice remaining in the fitting. A continuous white fibre was formed which could be drawn out of the coagulation bath. These fibres were then washed in PBS for at least 5 minutes to remove the remaining acid and then allowed to air dry.

2.3.4. Pilot scale fibre spinning

Two different spinning dopes were used for the large scale spinning trials. The first contained 9-10 % (w/v) total solids split between 46 mg/mL protein and 4.8 % sodium alginate (*laminaria hyperborea,* BDH). PEG present in the dope was not included in the solids calculation. The second had a much greater solids content, 140 mg/mL protein and 1 % sodium alginate. The sodium alginate was used to enhance the viscosity of the spinning solution and to reduce the self-adhesive properties of the spun protein fibres.

All spinning was carried out at room temperature and the operations were the same with the two types of dope. Estimation of protein concentration in the dope is described in Appendix 3.

For the low solids dope with equal concentrations of protein and alginate, 62 g of freshly thawed protein precipitate was added to 162 mL 6 M urea. Sodium alginate (10 g) was also added. Temperature was maintained at 37°C using a water bath and agitation was provided by a Eurostar basic mixer stirring at 500-700 rev / min (BDH) for 4-5 hours before being allowed to degas overnight at room temperature. Due to the presence of buffer in the initial precipitate the final volume of solution was approximately 210 mL and the final concentration of urea was 4.5 M.

The high protein dope was made with 281 g of freshly thawed precipitate dissolved in 100 mL 6 M urea. Sodium alginate (4.0 g) was also added. The final volume of solution was approximately 310 mL and the urea concentration had been reduced to 2 M.

The minimum volume of dope which could be used for each spinning trial was 200 mL.

This was placed in a stainless steel reservoir (total capacity 1 litre) and the dope was transferred by nitrogen pressure (15-40 p.s.i.) through a polythene tube, diameter 1 cm, to a flow control valve. When the polythene tube had filled the valve was opened and the dope passed through to a 1 mL/ rev. geared metering pump (Slack & Parr, Kegworth, UK). Dope was pumped at a rate of 10-20 mL/min depending on dope viscosity to the spinneret attachment point. When dope was seen to flow freely from the extrusion pipe, the spinneret with an orifice diameter of 1 mm and a channel length of 18 mm was screwed into place. This was then lowered into the coagulation bath (10 cm x 180 cm) containing 0.25 M HCl, 2 % $CaCl_2$, pH < 1.0.

Formed fibres were drawn by hand along the length of the coagulation bath, attached to a steel roller (diameter 10 cm, speed 21 rev/min) which collected the fibre from the coagulation bath and washed it in a water bath. A second set of rollers (diameter 10 cm, speed 31 rev/min) drew some of the fibres from the water bath into an acetone hardening bath from which the fibres were collected. Not all fibres were passed round both sets of rollers. This was because protein fibres are relatively weak and possess little wet strength and the spinning rig used was designed for the manufacture of much stronger synthetic fibres. Fibres were collected from either the water bath or the acetone bath and allowed to dry flat.

2.4. Mechanism of precipitation and materiai characterisation

2.4.1. Solubility curves

Solubility curves for a number of the fibronectin batches were determined, as well as fibronectin-rich or fibrinogen-rich solutions.

The pH of 4 mL samples of freshly thawed protein solution was lowered using 50 μL of either citric acid (concentrations 0.1 - 7.5 M) or hydrochloric acid (0.3 - 11 M) at either 22°C or 4°C. A Spiramix was used to agitate the samples for 15 minute before they were centrifuged for 15 minutes to remove any precipitate formed. The loss in sample volume caused by precipitation was measured for one solubility curve and was found to be 2.6 % at the pH of minimum protein solubility. This was regarded as small compared to the potential error in measuring such small volumes and so results are expressed

without a correction for lost precipitation volume. Total protein remaining in solution was measured using the Bradford protein assay.

2.4.2. Tensile testing

Samples of both drawn cables and spun fibres of length approximately 2 cm and diameter 100-800 μM were attached to a metal frame with polystyrene cross bars, using cyanoacrylate adhesive (RS Components Ltd, Corby, UK). To prevent sample contamination with the adhesive the cables were glued to the frame in a horizontal position and the glue allowed to dry before testing. The length of cable between the two points of attachment was then measured and taken as the sample gauge length. Samples were tested in a dry state using a 220M tensile testing rig, (The Testometric Company Ltd, Rochdale, UK) operating at a strain rate 2 mm/min. To test the in-cable variation in strength, tested samples were splinted and then retested ensuring that the point of breakage was clear of the glued region. Specimen dimensions were determined microscopically. Bulk protein material was made by compressing and blotting away surface water from undrawn protein precipitate before cutting it into 2 mm wide strips. Moist samples were tested in an enclosed damp environment by surrounding the testing frame with a plastic cylinder containing damp paper. The relative humidity was measured with a portable probe hygrometer (RS Components) and the moisture content of the material was also calculated using a dry weight method

Spun protein fibres could be handled much more easily than the fine drawn cables. For the fibres measurement of both fibre permanent set, the permanent increase in fibre length after testing, and elongation at break, the increase in fibre length at the point of breakage, could be made.

The flexibility of both spun fibres and drawn cables was compared in a method adapted from Huang *et al.* (1995). The method was based on the ability of fibres to bend around a curved object and gave a good indication of how easy the samples were to handle. Samples were wound round metal rods with diameters in the range 0.0625 to 0.5 inches and the diameter of rod at which they broke noted.

2.4.3. Moisture absorption - dry weight determination

Small, orientated blocks of material, 2 x 2 x 4 mm, were made by winding the freshly drawn cables around a fine glass rod into a mat. These were then either freeze dried (24 hours, -50°C, 60 mbar) or used after being freshly made. Standard relative humidity solutions were prepared, as described below and placed in a desiccator at 25°C. The change in mass of each mat was measured after the first 3 hours of incubation and then daily for 4 days. After 4 days there was no further detectable change in mass. Standard saturated solutions used were: lithium chloride, 15 % relative humidity (RH); calcium chloride 35 % RH; sodium nitrite, 65 % RH; sodium chloride, 76 % RH; potassium chloride, 86 % RH and sodium carbonate, 92 % RH.

Blocks of freeze dried material were hydrated repeatedly in both water and Dulbecco's minimal essential medium, (Gibco, Paisley, UK) at both room temperature and 37°C to assess the material's response to rehydration. Fluid uptake was measured as a change in mass based on the original wet weight.

To calculate the oven dry weight of a material, the sample was placed on a pre-weighed piece of filter paper (Whatman, Maidstone, UK) and the total mass measured. Sample plus filter paper were heated in an oven at 100°C until there was no further change in mass. The mass of the filter paper was subtracted from the initial and final masses and the change in mass in the sample calculated as being the original water content of the material.

Regain, the moisture content expressed as a percentage of the oven dry weight of the material, was calculated using equation 2.4.

$$R = 100\frac{w_w}{w_d} \qquad \text{(equation 2.4)}$$

To measure the swelling of spun fibres in solution, 2 cm lengths of fibre were loosely attached to glass microscope slides and placed in DMEM at 37°C. Fibre diameter was measured using a Nikon Profile Projector 6C-2 with a x 50 magnification before hydration and after 30 minutes, 1, 2, 3, and 4 hours.

2.4.4. Stability

Blocks of orientated material, similar to those used in the moisture absorption experiment were freeze dried, air dried at room temperature, dried in a 37°C oven or used freshly prepared. The blocks were incubated in 1 mL of water at room temperature with gentle agitation provided by a Spiramix. Samples were taken at 2, 4, 6, 8, 10, 20, 40 and 60 minute intervals and tested for protein content using the Bradford protein assay.

2.4.5. Yield and denier measurement

The mass and length of groups of 20 cables were measured for a total of 460 cables. The mass of 1 cm of cable was calculated and extrapolated to calculate the mass of 9000 metres of cable, or the denier. The length of the cable drawn per mL of fibronectin solution was also calculated.

2.4.6. Birefringence

Samples were viewed using a Reichert-Jung Polyvar-Met 66 widefield microscope (Cambridge Instruments Limited, Cambridge, UK) with a Leica polarizer (Leica, Milton Keynes, UK) placed beneath the stage. The angle of rotation of the polarizer to alter the lighting of the specimen from maximum illumination to total extinction was measured.

2.4.7. Scanning electron microscopy

Scanning electron microscopy was used to examine the surface of samples at high power. Wet samples had to be fixed, dehydrated, dried and sputter coated with gold before examination under the microscope. Dry samples were coated with gold without prior treatment.

The fixation and dehydration protocol is given in Appendix 1, 10.1.3. Samples were dried using hexamethyldisilazane (HMDS) liquid (Taab Laboratories Equipment Ltd., Aldermaston, Bucks, UK). Samples were covered with the solvent and allowed to air dry in a fume hood for 30 minutes. Excess solvent was then removed and the samples were allowed to air dry overnight before gold sputter coating using an Emscope SC500

sputter coater (Emscope Laboratories Ltd, Ashford, UK). Samples were examined using a ISI100A electron microscope at 60-75 μA (International Scientific Instruments Inc., Santa Clara, California, USA).

2.4.8. Image analysis

Cable samples were fixed for 3 hours at 4°C in 2.5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer before staining for 10 minutes with 1 % toludine blue solution and rinsing with distilled water. An image analysis system with general imaging software package (Genias), (Joyce-Loebel, Tyne and Wear, UK) was used to measure component fibres of the main cable.

2.5. Fibronectin cables on backing materials

Drawn fibronectin cables were attached to collagen sponges to qualitatively examine the effect of the cables orientating cells on a biomaterial.

2.5.1. Human dermal fibroblast cell culture

Human dermal fibroblast explants were taken post-surgically from skin samples. Small cubes (2 mm^3) of clean, washed skin from abdominoplasty surgery were placed in 25 cm^2 tissue culture flasks and fed with Dulbecco's minimal eagle medium supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin (Life Technologies Ltd, Paisley, UK) and 10 % (v/v) foetal calf serum (First Link UK Ltd, West Midlands, UK). After 3-4 weeks fibroblasts had migrated from the explants and had attached to the flask in a confluent sheet These cells were passaged every 2-3 weeks with a 1:8 increase in total area each time. A trypsin-EDTA solution in PBS (Life Technologies) was used to detach the cells from the culture flask. Cells were used between their 2nd and 7th passage.

2.5.2. Preparation of collagen sponges / fibronectin cables

Collagen sponges, 7 x 5 x 0.6 cm, which had been cross-linked by diphenylphosphorylazide, DPPA, (Coletica, Lyon, France) to improve their stability

(Petite *et al.,* 1994; Rault *et al.*, 1996) were used as a backing material for human dermal fibroblast attachment, as a test for the formation of an orientated sheet of cells. The sponges were attached to a teflon roller which could be rotated at a constant speed by a motor and also precessed at a rate of 1 mm/rev.

Fibronectin cables were made as described in section 2.3.2. and drawn directly onto the collagen sponge from solution. A glass rod was used to guide the cables onto the sponge and to assist the laying down of evenly spaced cables whilst the sponge rotated at a rate of 6 rev/min and also precessed.

The sponges with cables attached were washed for 15 minutes in PBS and then freeze dried before being cut into 1 cm^2 segments and sterilised by gamma irradiation for 48 hours - maximum exposure approximately 3 MR.

Sponges were placed 1 per well in a 12 well plate (Life Technologies Ltd., Paisley, Scotland) with 0.5 mL of DMEM supplemented with 50 μg/mL ascorbic acid to assist collagen production by the fibroblasts and incubated at 37°C for 30 minutes. The aim of the experiment was to observe cell attachment to the surface of the sponge, so cells were only seeded onto pre-moistened sponges. Sabbagh (1997) noted that seeding cells onto a dry sponge resulted in the cells being drawn down into the centre of the sponge whilst seeding onto a wet sponge enhanced cell attachment to the sponge surface. Sponges were seeded with 300 000 cells in 0.5 mL media corresponding with work done by Doillon *et al.* (1987) and Berthod *et al.* (1993; 1996) whilst control sponges were left without cells. Cells were also seeded onto sponges without fibronectin cables attached. Samples were left in a 37°C incubator with 5 % CO_2 atmosphere for 30 minutes to allow the cells to attach to the sponge before a further 0.5 mL media was added. Samples were then left for 1, 3, 7 and 14 days with the medium being changed daily. Sponges were fixed at the end of their time course with 2.5 % glutaraldehyde in phosphate buffer overnight and processed for examination by SEM. Pore size of the collagen sponge was measured in an attempt to express quantitatively the attachment of human dermal fibroblasts to the sponges.

3.0. The production and characterisation of fibronectin for fibre preparation

3.1. Introduction

Previously affinity chromatography purified human plasma fibronectin (Bio-Products Laboratory, BPL, Elstree) has been used to make cell guidance scaffolds in the form of mats, using a bench-scale method (Ejim *et al.*, 1993, Brown *et al.*, 1994). The aim of the work described in this thesis was to use fibronectin from an alternative source (Protein Fractionation Centre, PFC, Edinburgh) to make scaffolds for orientating cells but on a larger scale.

This chapter describes the transfer and development of assays for measurement of protein components in the donated fibronectin-rich solutions. These assays were then used to mass balance the process of fibronectin extraction from cryoprecipitate and detect variations between batches. The cryoprecipitate-derived solutions were used to examine the fibronectin mat making process and assess its suitability for scale-up.

3.2. Assay development

When purified protein solutions were used to make fibronectin mats, the concentration of protein in solution could be estimated by measuring the absorbence of the solution at 280 nm. The absorbence could then be converted to a concentration using the extinction coefficient for fibronectin. Unfortunately when applied to mixtures of proteins, this procedure gives no indication of the proportion of each protein. Measurements of plasma levels of fibronectin and fibrinogen are made in clinical practice as an essential part of the diagnosis of certain disease states. Salonen *et al.* (1984) and Gomez-Lechon and Castell (1985, 1986) describe ELISAs for the measurement of human plasma fibronectin whilst Parfentjev *et al.* (1953), Exner *et al.* (1979) and Nieuwenhuizen (1995) describe methods for the measurement of fibrinogen concentration. However the concentrations of fibronectin and fibrinogen in human plasma are of the order of 0.3

mg/mL (McKeown-Longo and Mosher, 1989) and 2-4 mg/mL (Doolittle, 1975), thus the assays were tested to see if they could be used for the fibronectin-rich solution available for this work.

3.2.1. ELISA for fibronectin

The ELISA method used is described in 2.2.2. and a standard curve is shown in 10.1.1.2. A number of experiments were carried out during the development of the fibronectin ELISA to choose an appropriate plate coating buffer, suitable antibody concentrations, incubation time and controls.

3.2.1.1. Coating buffer

Standards were diluted either in 0.01 M phosphate-buffered saline, pH 7.4 or in a 0.015 M sodium carbonate / 0.035 M sodium hydrogen carbonate buffer, pH 9.6. Both sets of standards bound to the ELISA plate to produce a standard curve on completion of the assay. However phosphate-buffered saline was used preferentially as the coating buffer because the fibronectin was already prepared in a phosphate based buffer.

3.2.1.2. Antibody concentrations

Two antibodies were used for the fibronectin ELISA. A rabbit anti-human fibronectin antibody (1st antibody) which attached to the fibronectin bound to the plate and a goat anti-rabbit alkaline phosphatase linked IgG (2nd antibody). To select appropriate dilutions of both antibodies, the absorbence of samples at the concentrated end of the standard curve, 2-8 μg/mL, were measured with different combinations of antibody dilutions. The 1st antibody was added at either 4, 6, 8, 10 or 12 000 times dilutions whilst the second was used at 2, 4, 6, 8 or 10 000 times dilution. Samples which were more concentrated than the assay range (10-30 μg/mL) were included to demonstrate a maximum absorbence and highlight the highest concentration the assay was sensitive to. Background measurements were made to test whether there was an interaction between the two antibodies at high concentrations by incubating both antibodies in the absence of fibronectin. An ideal situation was considered as an absorbence of 1.0 for an 8 μg/mL solution and a background reading of zero for the two antibodies in the absence of fibronectin. First antibody dilutions of 4 000 and 6 000 times gave background

absorbence readings greater than 0.7 and 0.5 when combined with a second antibody diluted 6 000 times and were therefore too concentrated for use in this assay. The 8, 10 and 12 000 times diluted antibodies had background values of zero and all demonstrated a decrease in absorbence as the fibronectin concentration was lowered into the assay range, Figure 3.1. However these 8, 10 and 12 000 times first antibody dilutions when coupled with a 6 000 times dilution of the second antibody gave absorbences of only 0.65 and 0.8 for an 8 μg/mL solution and so the effects of altering the second antibody concentration were considered. The effect of holding the first antibody concentration at a 10 000 dilution and altering the concentration of the 2nd antibody is shown in Figure 3.2. All these combinations had a background level of zero. A 2 000 times dilution did not produce a distinct decrease in absorbence as the fibronectin concentration was lowered into the assay range, whilst the decrease with a 12 000 times dilution was less distinct than 4-8 000 times dilutions. A combination of 10 000 times dilution for the first antibody and 4 000 times dilution for the second antibody was chosen because an absorbence of approximately 1.0 was given for values at the top end of the assay range. The second antibody required storage at 4°C and it was noticed that an 8 000 dilution could be used satisfactorily for this antibody shortly after purchase but after storage at 4°C for longer than 3 months, a 4 000 times dilution produced more successful results.

3.2.1.3. Incubation time

The change in absorbence of the concentrated end of the standard curve, incubated at 30°C, was observed after 15, 30, 45, 60 and 120 minutes and the results shown in Figure 3.3. A colour change was observed after 15 minutes although it did not distinguish the start of the assay range from the maximum absorbence effectively. Readings after 30, 45, 60 and 120 minutes all showed a distinction between the sample in range and those not, however 1 hour incubations were preferred because they gave a reading of approximately 1.0 at the most concentrated end of the assay range.

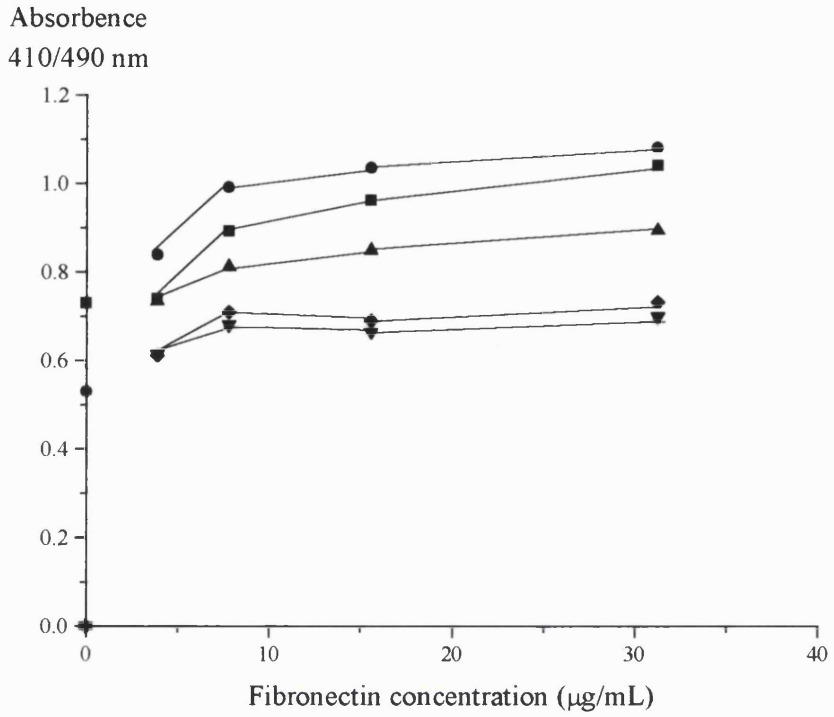


Figure 3.1. *The effect of altering the concentration of the 1st antibody on absorbence. The 2nd antibody was always diluted 6 000 times and plates were incubated for 1 hour at 30°C. Dilution of 1st antibody :* ■ *- 4 000,* ● *- 6 000,* ▲ *- 8 000,* ▼ *- 10 000,* ◆*- 12 000.*

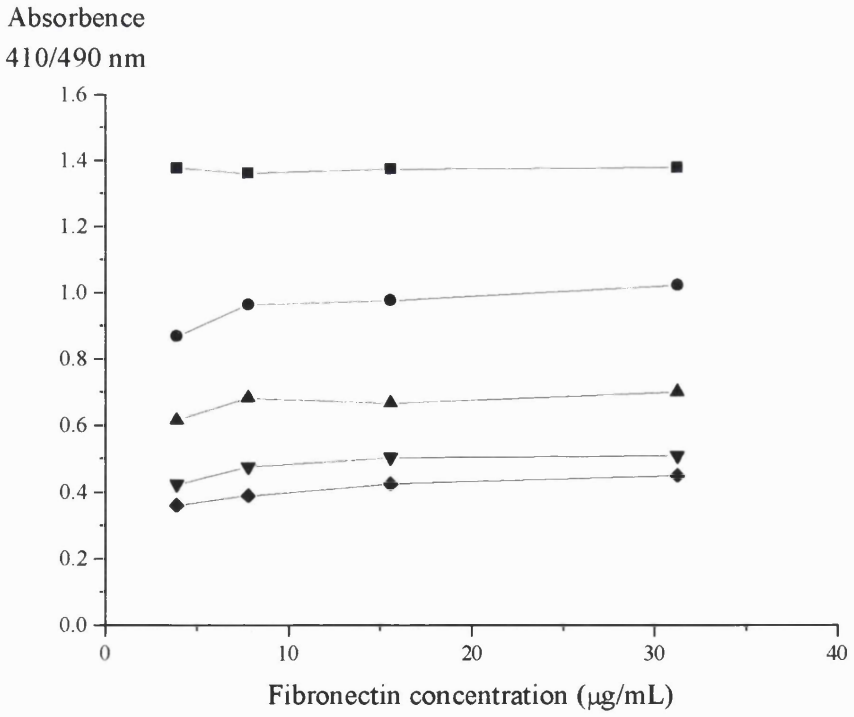


Figure 3.2. *The effect of altering the concentration of the 2nd antibody on absorbence. The 1st antibody was always diluted 10 000 times and plates were incubated for 1 hour at 30°C. Dilution of 2nd antibody :* ■ *- 2 000,* ● *- 4 000,* ▲ *- 6 000,* ▼ *- 8 000,* ◆*- 10 000.*

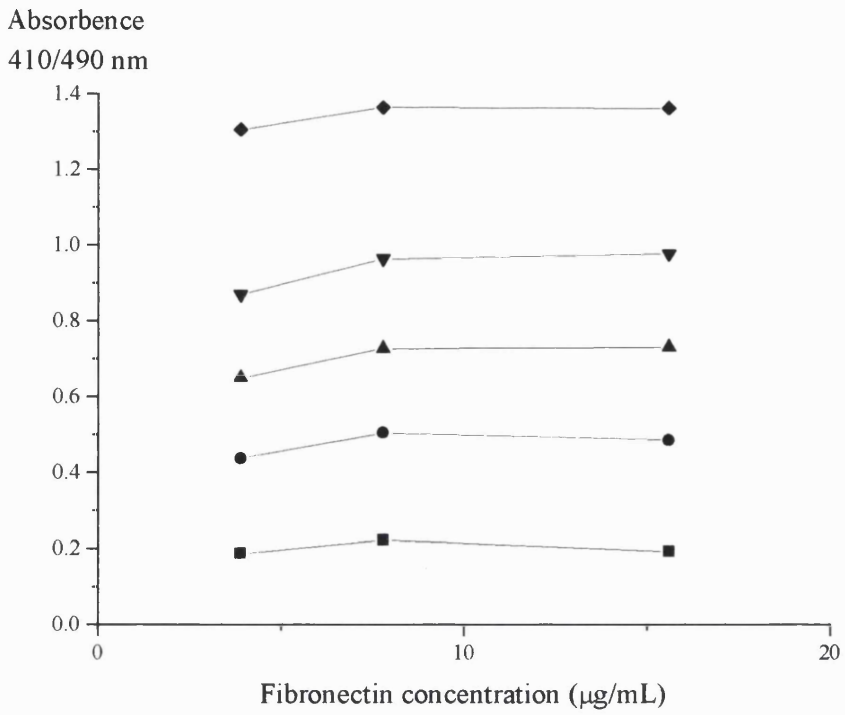


Figure 3.3. *Comparison of absorbence for the concentrated end of the assay range with increasing incubation time. Plates were incubated at 30°C after the addition of enzyme substrate, pNpp. Antibody dilutions used were 10 000 and 4 000 for the 1st and 2nd antibodies respectively. Incubation time:* ■ *- 15 mins;* ● *- 30 mins;* ▲*- 45 mins;* ▼ *- 60 mins;* ◆ *- 120 mins.*

3.2.1.4. Controls and cross-reactivity

To test that the change in absorbence measured was proportional to the quantity of fibronectin bound to the well, a number of controls were used. A blocking solution, containing albumin, was used to prevent non-specific binding of the antibodies to the plastic. It was therefore important to assess whether the antibodies bound to the albumin. Figure 3.4 shows the absorbence seen for controls and samples. From this data it was seen that fibronectin (concentration 1.6 μg/mL) and both first and second antibodies were required for a significant colour change to occur. The second antibody does not appear to bind to the blocking buffer and consequently it was assumed that there was no cross-reactivity with albumin.

Cross-reactivity of the antibodies with fibrinogen was also tested using two sources of human fibronectin. Two samples of fibrinogen, one purchased from Sigma and the other donated by Protein Fractionation Centre, were examined by gel electrophoresis for

contamination with fibronectin. These samples can be seen in Figure 3.5 (lanes 4 and 5). The sample donated by PFC contains some fibronectin, although it has been estimated as less than 5 % of the total protein present (A.J. MacLeod, personal communication), whilst the Sigma sample appeared to contain little. Protein fragments, including fibronectin, may be present but are indistinguishable on the gel. Testing these samples with the fibronectin ELISA, fibronectin was seen to constitute 29 % ± 7 (95 % confidence interval) of the total protein in the PFC sample and 12 % ± 15 % in the Sigma samples, contradicting results shown by the gel. Thus it appears that there may be some cross-reactivity between the antibodies and the fibrinogen in solution. The ELISA has mainly be used examine the change in fibronectin concentration in solution with changing environmental conditions. As this requires the measurement of relative values, any cross-reactivity will not affect the results significantly. Absolute measurements of fibronectin have also been made for each batch of fibronectin produced and the results of these are similar to those established by in-house methods at PFC.

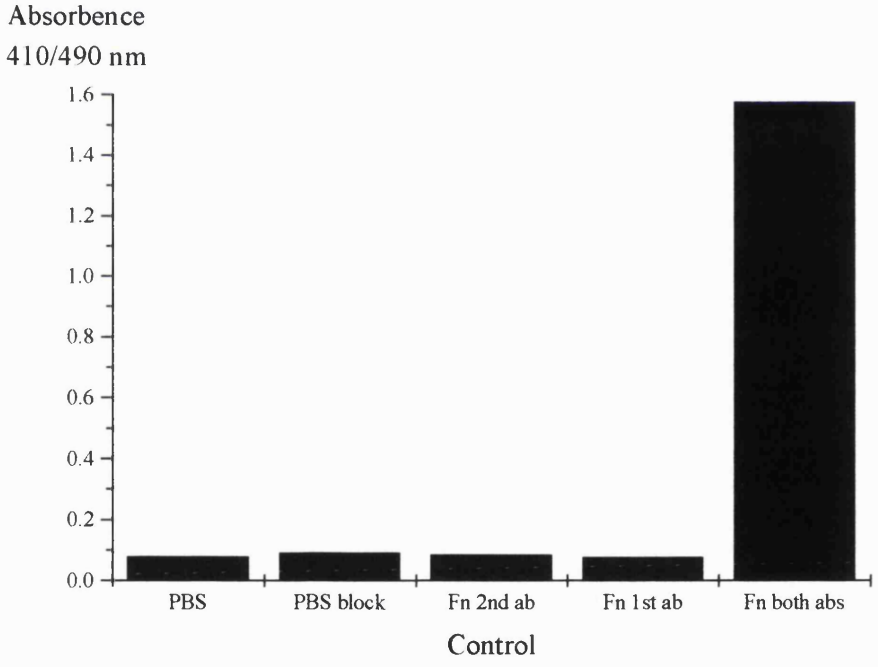


Figure 3.4. *Absorbence values for ELISA controls. From left to right PBS - no fibronectin bound to well; PBS block - blocking solution bound to well; Fn 2nd ab - fibronectin with blocking solution and second antibody conjugate; Fn 1st ab - fibronectin with blocking solution and first antibody; Fn both abs - fibronectin with blocking solution and both antibodies. Alkaline phosphatase substrate, 2 mg/mL, was added to each well. The fibronectin concentration was 1.6 μg/mL*

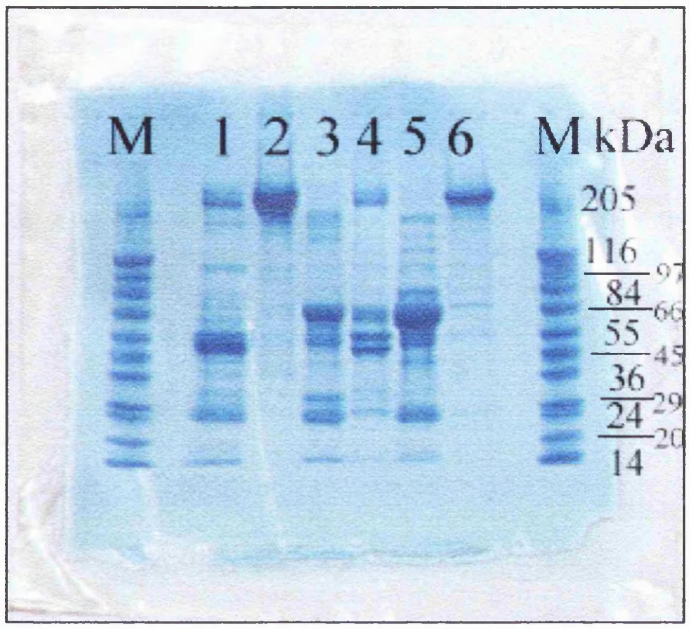


Figure 3.5. *SDS electrophoretic gel demonstrating samples from affinity chromatography, total protein, fibrinogen and fibronectin standards. Tris-glycine 8-16 % gradient gels were used with 20 µg total protein loaded into each well.*

Fibronectin - MW 215 and 220 kDa
Fibrinogen - MW 45, 55 and 65 kDa
Albumin - MW 66kDa

Lane M	Wide range molecular weight markers (see 10.1.2)
Lane 1	Breakthrough from affinity chromatography column
Lane 2	Eluted fraction from chromatography column
Lane 3	Albumin / globulin standards used for total protein assay
Lane 4	PFC fibrinogen standards
Lane 5	Sigma fibrinogen standards
Lane 6	Purified fibronectin

3.2.2. Fibrinogen assay

The favoured method for estimation of fibrinogen concentration is the measurement of clotting time after thrombin addition to a fibrinogen sample. However this method is known to be both time consuming and technically demanding (Exner *et al.*, 1979) so two alternative methods have been examined.

The heat precipitation assay was transferred from PFC, Edinburgh whilst the ammonium sulphate salt precipitation method was performed according to Fowell's adaptation of the method of Parfentjev *et al.* (1953). Briefly, samples were diluted 10 fold with 1 M ammonium sulphate, 0.27 M sodium chloride, pH 7.0, mixed and left for 3 minutes. The absorbence was measured at 510 nm. Both methods were tested using fibrinogen standards, 0.5 -3.0 mg/mL. Standard curves are shown in Figure 3.6.

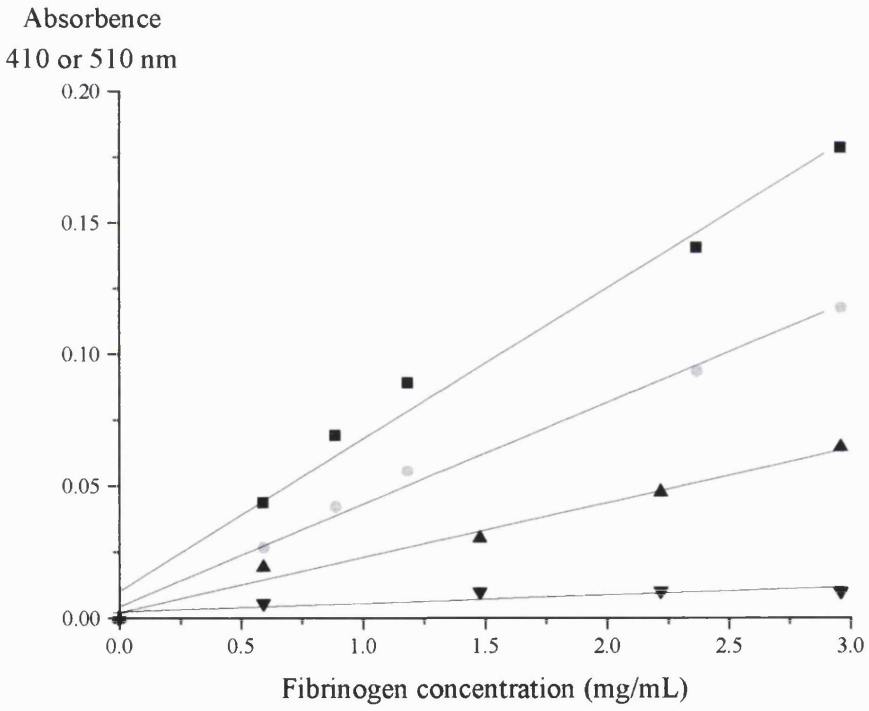


Figure 3.6. *Fibrinogen calibration curves for the two tested fibrinogen assays. Heat precipitation assay:* ■ *- samples diluted 1/2;* ▲ *- samples diluted 1/10. Ammonium sulphate precipitation assay,* ● *- samples diluted 1/2;* ▼*- samples diluted 1/10. Standards used were from Sigma Chemical Co. and were diluted into assay range using phosphate buffered saline before dilution with the appropriate assay buffer.*

3.2.2.1. Sample dilution.

When samples were diluted 1/10 with the appropriate assay buffer, absorbence measurements were low, <0.05, in the concentration range of interest, 0.5-2 mg/mL. To increase the sensitivity of the assay, 1/2 dilutions were made to give absorbence readings in the range 0.05 - 0.2 (Figure 3.6). The heat precipitation assay was preferred to the ammonium sulphate assay because of its greater sensitivity within the concentration range of interest.

3.2.2.2. Standards and cross- reactivity

The reagent blank consisted of phosphate buffer and assay mix in a 50/50 (v/v) mixture. A calibration curve constructed using Sigma fibrinogen reference standards is compared to one constructed using PFC fibrinogen in Figure 3.7. The PFC standards gave higher absorbencies for a given fibrinogen concentration than the Sigma standards.

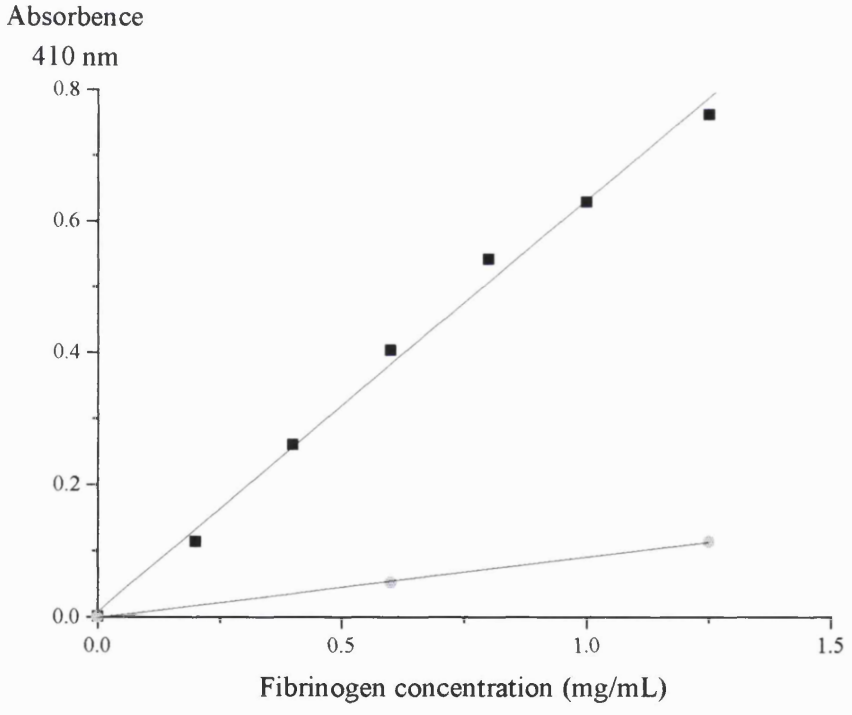


Figure 3.7. *Standard curves for the heat precipitation assay using two different sets of fibrinogen standard,* ■ *- PFC fibrinogen;* ● *Sigma fibrinogen. All standards were diluted 1/2 with assay buffer.*

Comparing the composition of the two standards from the electrophoretic gel, Figure 3.5, lanes 4 and 5, the PFC sample contained both fibronectin and fibrinogen, with fibrinogen clearly represented by its 3 characteristic bands. The Sigma sample, lane 5, contained fibrinogen, but the 3 bands, representing the 3 chains, did not appear in equal proportion. Albumin was also present in both samples, which made the fibrinogen A chain band (65 kDa) appear diffuse. There appeared to be most albumin in the Sigma standard, which gave the appearance of out of proportion fibrinogen chains. There was little fibronectin present but smaller molecular weight proteins, 6-14 kDa could be identified. These could be protein fragments or other small, unidentified molecular weight proteins. These differences in composition could affect the standard's precipitation behaviour. Since the composition of the PFC standards bore greater similarity to the cryoprecipitate-derived solutions being used, the fibrinogen assay was carried out with PFC standards with 1/2 dilutions for both standards and samples. A typical calibration curve is shown in 10.1.1.3. The absorbence of a purified fibronectin solution tested using the heat precipitation assay was similar to the reagent blank and thus showed no cross-reactivity.

3.2.3. Total protein assay

The dye based Bradford protein assay was chosen for its ease of use and the absence of many chemical interferences, including with 6 M urea. However the development of colour on contact with protein is known to be greater with albumin, commonly used as a standard, than with other proteins, e.g. γ globulins.

Standard curves were prepared, concentrations 0.1-1.0 mg/mL, using albumin, purified fibronectin and an albumin / globulin mixture (Sigma). Purified fibronectin was seen to give a lower absorbence than both the other two solutions, for a given total protein concentration (Figure 3.8). Since the concentration of purified fibronectin solutions was usually measured using the absorbence at 280 nm and only the protein mixtures were measured with the dye binding assay, the albumin / globulin standards were taken as the most appropriate. The albumin / globulin standards were examined by gel electrophoresis (Figure 3.5, lane 3) and seen to contain both fibrinogen and albumin, as well as other small molecular weight proteins, but not fibronectin.

The recommended wavelength for measuring the dye colour for the protein assay was 595 nm but to measure the protein content of many samples at once, a plate reader with a 570 nm filter was used. To ensure that this did not adversely affect the quality of the assay, calibration curves using albumin/globulin standards and purified fibronectin were carried out at 595 nm using a spectrophotometer and 570 nm using the plate reader (Figure 3.9). Although the curves were shifted using 570 nm as the reading wavelength for the assay, it did not affect the linear calibration between 0.1 - 1.0 mg/mL.

The polyethylene glycol assay was transferred from PFC without alteration and the albumin assay was according to the method of Kaplan and Szabo (1983).

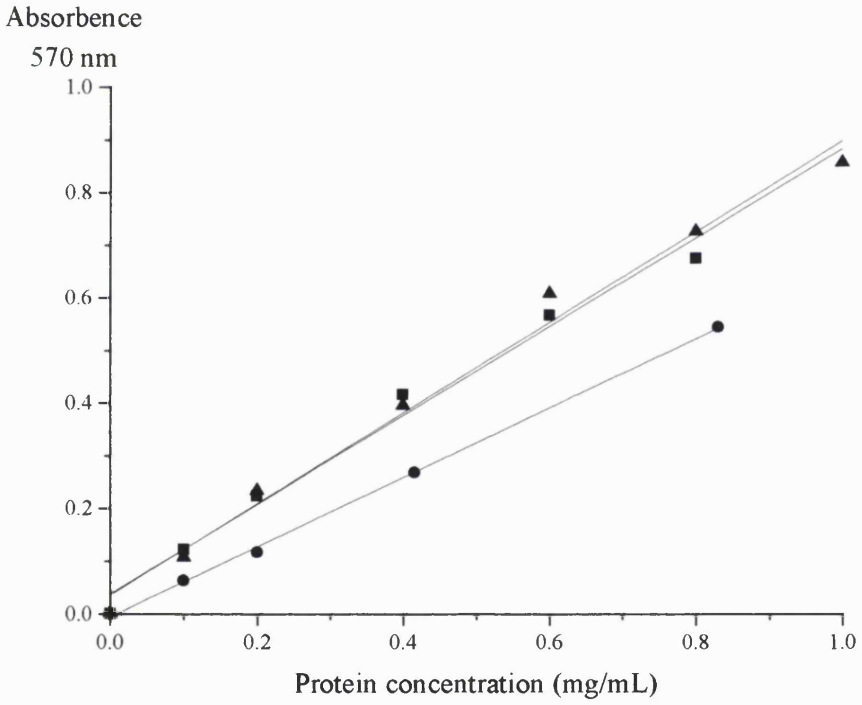


Figure 3.8. Testing calibration standards with the Bradford protein assay, ■ *- bovine serum albumin;* ● *- purified fibronectin;* ▲ *- albumin / globulin standards.*

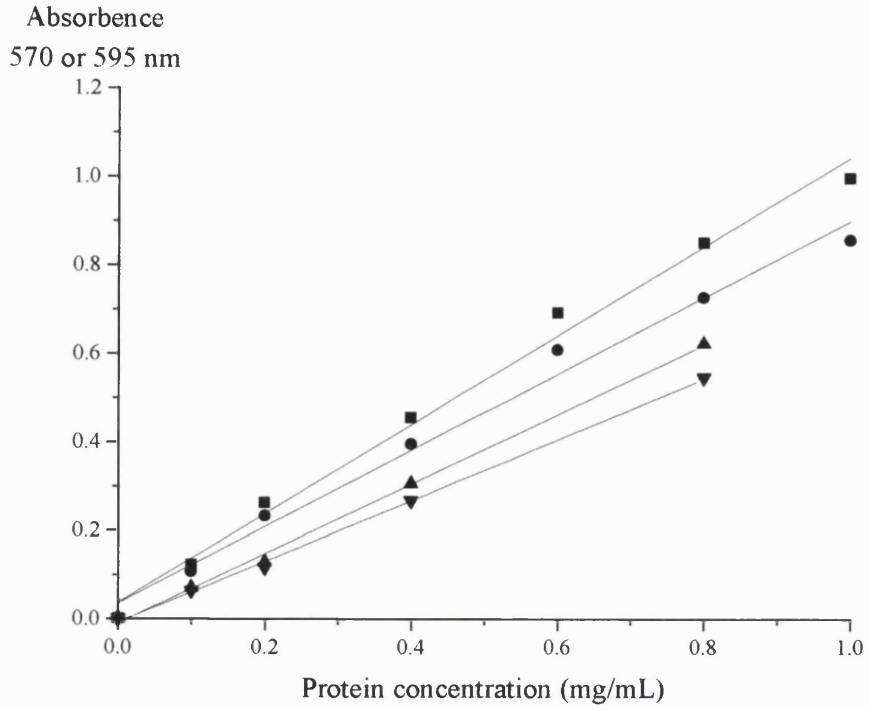


Figure 3.9. *Calibration curves for albumin / globulin solutions, read at ■ - 595 nm; ● - 570 nm and purified fibronectin solutions at ▲ - 595 nm; ▼ - 570 nm.*

3.3. Solution production and characterisation

Fibronectin enriched solutions were produced from plasma cryoprecipitate, using a PEG fractionation technique, described in section 2.1. A number of batches were prepared during the course of this work and were labelled with a 3 figure code. The same codes have been used in this section to identify and discuss individual batches.

3.3.1. Mass balancing the production process

A detailed examination of the fibronectin extraction process for batch 010 is shown in Figure 3.10. The first precipitation (4 % w/v PEG) removed 93 % of the fibrinogen from the solution and 58 % of the fibronectin. The subsequent 10 % PEG precipitation removed the remaining proteins from solution leaving < 2 g protein in the supernatant. Assay results showed more PEG (890 g) in the 1st supernatant than had originally been added (782 g). However the maximum error of the PEG assay has been calculated as 29 % (section 10.1.1.4), thus this difference was attributed to assay error. The final PEG/protein ratio was measured as 0.3.

Inter-batch variations are discussed in section 3.3.3

3.3.2. Solution characterisation

The protein composition of the fibronectin-enriched solutions was examined qualitatively using gel electrophoresis (Figure 3.11) and quantitatively by immuno-assay for fibronectin and precipitation assay for fibrinogen. The gel, which is also discussed in section 3.3.3, demonstrated that the solutions contained more constituents than the fibronectin (2 bands ~ 220 kDa) and fibrinogen (3 bands ~ 45-65 kDa). Proteins such as albumin, molecular weight 66 kDa, were known to be present as well as fibronectin and fibrinogen molecules which have been degraded. Batch 012 was stored at 4°C for six months before running on the gel and appeared to contain a high proportion of small molecular weight proteins and showed only light banding at the 220 kDa level, where fibronectin would be expected to be seen. This suggested that the fibronectin and to some extent also the fibrinogen had been broken down during storage and was attributed to bacterial degradation. Other electrophoretic gels confirmed that there had been fibronectin and fibrinogen present in an intact state before storage.

Miekka (1983) noted that extra bands are present on fibronectin gels after samples were heated to 100°C during their preparation. Bands were particularly noticeable at 65 kDa and 100 kDa although smaller bands appeared throughout, suggesting that some of the bands seen in Figure 3.11, which cannot directly attributed to either fibronectin or fibrinogen, may be due to temperature induced degradation.

Assays were used to detect and quantify the amount of fibronectin, fibrinogen, albumin and PEG in the final solution or precipitate. The concentration of two other plasma proteins, thrombin and factor XIII were measured at PFC. The final composition of two batches is shown in Table 3.1. For batch 012, a 59 %:41 % fibronectin / fibrinogen ratio has also been measured. PEG / protein ratios of between 0.4 - 0.6 have been recorded and this variation may depend on how much liquor was associated with the precipitate when it was dissolved.

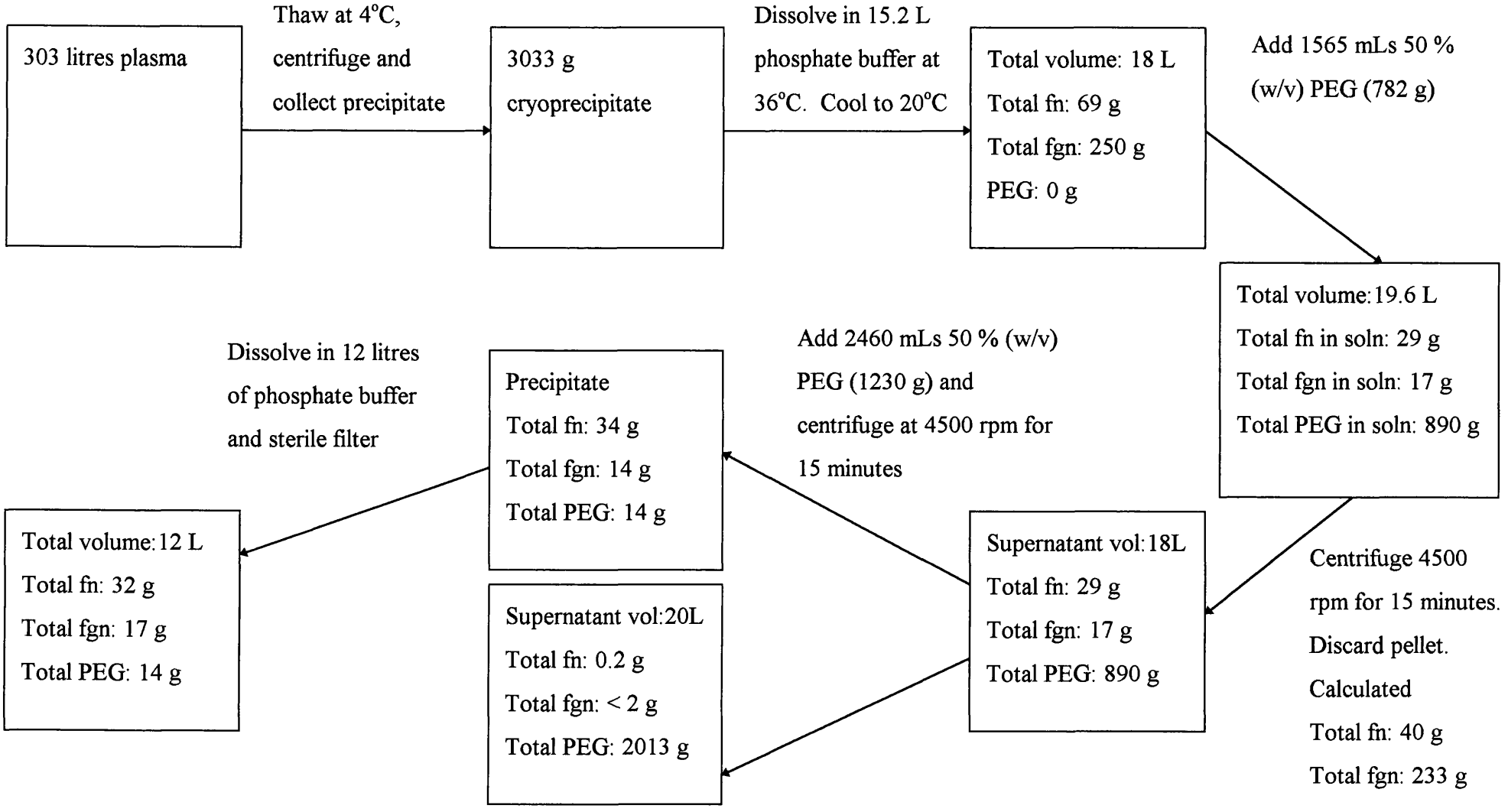


Figure 3.10. *Process flowsheet for manufacture of batch 010 fibronectin-enriched solution from plasma. Operational experience at the PFC has shown that about 10 g of cryoprecipitate could be extracted from 1 litre of plasma. Amounts of protein / polyethylene glycol have been measured by assay unless they are labelled as calculated values. Fn=fibronectin; fgn=fibrinogen.*

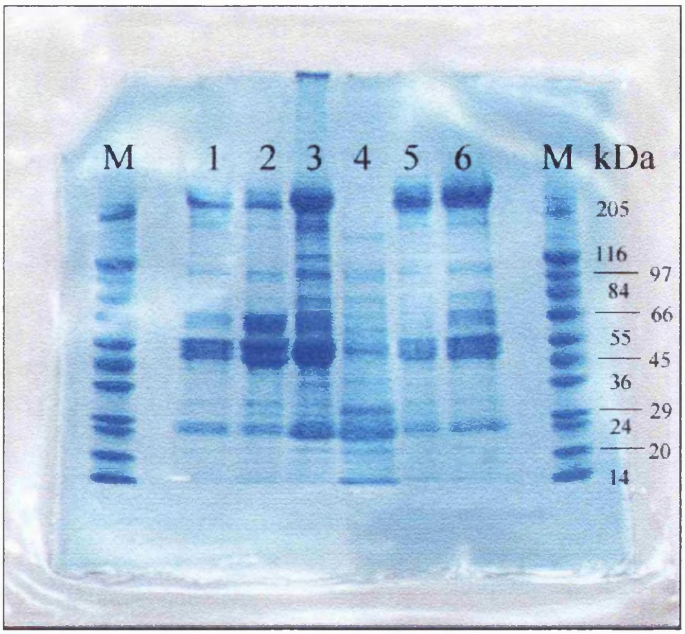


Figure 3.11. *SDS electrophoretic gel demonstrating protein constituents of 6 batches of fibronectin / fibrinogen solution. Tris-glycine 8-16 % gradient gels were used with 20μg total protein loaded into each well. All samples were stored frozen until run on the gel except sample 012, which was stored at 4°C and showed signs of protein degradation.*

Fibronectin chains - MW 215 and 220 kDa
Fibrinogen chains - MW 45, 55 and 65 kDa
Albumin - MW 66 kDa

Lane M Wide range molecular weight markers (see section 10.1.2)

Lane 1 Batch 017

Lane 2 Batch 014

Lane 3 Batch 013

Lane 4 Batch 012

Lane 5 Batch 011

Lane 6 Batch 010

Batch	011	012
Total protein (mg/mL) ± 5 %	4.7	16.0
Fibronectin (mg/mL) ± 14 %	3.4 (72)	14.4 (74)
Fibrinogen (mg/mL) ± 16 %	1.3 (28)	5.1(26)
Albumin (mg/mL) ± 20 %	0.4-0.9	2.3
Polyethyleneglycol (mg/mL) ± 29 %	3.0	10.0
Thrombin (U/mL)	<0.015	-
Factor XII (U/mL)	<0.1	-
PEG/protein	0.6	0.6

Table 3.1. *Composition of two batches of fibronectin / fibrinogen. Fibronectin was found to interfere with the albumin assay so measurements given are approximate and have taken into account some cross-reactivity. Figures in brackets represent % total protein. U/mL represents one unit of a factor or protein present in 1 mL of a pool of human plasma. The PEG protein ratio is expressed as a ratio of dry weight of the components present. Batch 011 was prepared as a solution, with total protein 4.7 mg/mL, dissolved in 0.01 M $NaH_2PO_4.2H_2O$, 0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.0. Batch 012 was prepared as a precipitate and measurements of the components of the precipitate were made after 0.55 g of precipitate was dissolved in 5 mL of the phosphate buffer used for batch 011. The calculated maximum % error for the appropriate assay is given in the first column.*

3.3.3. Fibronectin : Fibrinogen batch variations

Batch to batch variations in the protein composition of the final PEG precipitate in six different batches were examined and the results summarized in Table 3.2. Operational experience at the PFC has shown that about 10 g of cryoprecipitate could be extracted per litre of donated plasma and this figure will be used for these calculations. Assay of fibronectin and fibrinogen concentrations were made at PFC. In all six cases 46-60 % of the fibronectin present at the start of the process remained in the precipitate after the two PEG cuts. Processing a small batch of cryoprecipitate, 489 g (batch 007) compared to 4500 g (batch 012), did not alter the final composition of the precipitate. For five batches the amount of fibrinogen remaining after the precipitation process was 6-12 % of the original level. This was not the case for batch 014, which had 40 % of the initial level left at the end and a final fibronectin : fibrinogen ratio of 25:75. All other

batches contained more fibronectin than fibrinogen after processing. The initial fibrinogen content of the cyroprecipitate for batch 014 was not greater than for the other batches. The initial fibrinogen content of the cryoprecipitate in all cases was 4.7 - 8.8 % (w/w), whilst for fibronectin it varied from 1.9-2.6 %. The fibronectin yield per litre of donated plasma ranged from 0.10 - 0.15 g/L whereas for fibrinogen the range was from 0.05 - 0.11 for the low fibrinogen batches and 0.34 for the high fibrinogen batch. Thus it appears that for batch 014 there was a failure to remove the majority of the fibrinogen rather than an excess removal of fibronectin.

The electrophoretic gel, shown in Figure 3.11, shows 6 batches of fibronectin-enriched solution, 010, 011,012, 013, 014 and 017. It can be seen from the gel that batches 013 and 017 have bands representing fibronectin and fibrinogen with densities in the same proportion as batches 010 and 011, indicating the two proteins are present in consistent proportions between batches. Batch 012 was described in section 3.3.2. Batch 014 shows a high density of the three bands characteristic for fibrinogen and a lower density for the fibronectin bands.

The concentration of fibronectin in plasma is about 0.3 g/L, although considerable variation is seen between individuals. Approximately 60 % of the fibronectin in plasma enters the cyroprecipitate fraction on thawing (Horowitz and Chang, 1989) and thus a one litre plasma donation (10 g cryoprecipitate) would be expected to yield approximately 0.18 g fibronectin after cryoprecipitation. The intial fibronectin content of 10 g cryoprecipitate was measured as between 0.16 and 0.26 g for the six batches examined, demonstrating variation in either the inital plasma pool or the proportion of fibronectin which enters the cryoprecipitate fraction. Using the measured values of fibronectin initially present in the cryoprecipitate and the fibronectin yield after PEG fractionation, the recovery of fibronectin initially present was 45-69 %.

The poor removal of fibrinogen from batch 014 was examined further. After the 4 % PEG cut 68 % of the initial fibrinogen was still in solution as well as 81 % of the fibronectin. From batch 010 (Figure 3.10) it was noted that 7 % and 42 % of the two proteins were left at the same stage. Thus it was concluded that there was a failure in the first precipitation. It has been recorded that the amount of fibrinogen in cyroprecipitate may be altered by varying the temperature of plasma during thawing, (Farrugia *et al.*,

1992) and consequently the temperature of thawing of the frozen cryoprecipitate may also be important. Batch 014 was thawed for 3 days at 4°C before processing, whilst batch 010 was only chilled overnight. It may be necessary to investigate temperature control throughout the process, including temperature of initial dissolution and cooling before PEG addition.

3.3.3.1. Further PEG enrichment of high fibrinogen batches

The high fibrinogen batch was redissolved in phosphate buffer and two further PEG cuts were made at concentrations of 4 % and 10 %. Two batches were examined and in both cases at least 85 % of the fibrinogen was removed in the first PEG precipitation and the final precipitate consisted of 25-37 % fibrinogen and 63-75 % fibronectin. This represented a PEG fractionation typical of the majority of batches.

Batch Ref	cryo processed (g)	source plasma (L)	remaining fn after process (%)	remaining fgn after process (%)	fn:fgn ratio at start (%)	fn:fgn ratio at end (%)	fn yield / L plasma (g/L)	fgn yield / L plasma (g/L)	initial fn content of 10 g cryo	initial fgn content of 10 g cryo
007	489	49	50	8	20:80	61:39	0.10	0.07	0.21	0.82
009	3146	315	55	12	32:68	67:33	0.12	0.06	0.22	0.47
010	3032	303	46	7	21:79	66:34	0.10	0.05	0.22	0.83
011	4324	432	53	6	22:78	72:28	0.11	0.04	0.21	0.73
012	4500	450	59	12	23:77	59:41	0.15	0.11	0.26	0.88
014	5123	512	70	47	18:82	25:75	0.11	0.34	0.16	0.73

Table 3.2. *Details of fibronectin and fibrinogen content of the final PEG precipitate for 6 batches of processed cryoprecipitate. Protein yield / L plasma was calculated from the mass of protein extracted / L of plasma initially donated. Initial protein content of cryoprecipitate was calculated from the inital mass of the specific protein /L of plasma donation. The initial fibronectin content of 10 g of cryoprecipitate is equivalent to fibronectin from 1 L of plasma donation entering the process.*

3.4. Affinity chromatography purification of fibronectin

Affinity chromatography was used to purify fibronectin from the fibronectin-fibrinogen solution. The column used was loaded with 25 mL of gelatin Sepharose (pre-conjugated, Sigma), with a bed height of 12.5 cm and a cross-sectional area of 2 cm^2. The total gelatin in the column was in the range 75-150 mg (Sigma). A typical loading - elution profile is shown in Figure 3.12. The first peak represents fibrinogen passing through the column without binding. Since the protein content of the first fractions collected was greater than the initial fibrinogen content of the solution, fibronectin which had not bound to the column must also have passed through the column. Sample 1, when examined using gel electrophoresis, (figure 3.5, lane 1) contained mainly fibrinogen but also some fibronectin, suggesting that the amount of fibronectin added exceeded the column binding capacity or the fibronectin affinity for the column was poor. Elution with 4 M urea resulted in the removal of 84 % of the bound fibronectin. Sample 2, when taken and run on the gel (figure 3.5, lane 2), demonstrated that the main component of the eluant was fibronectin, with smaller amounts of other proteins or fibronectin degradation peptides. A number of batches of fibronectin were purified and the results are summarised in Table 3.3.

Regnault *et al.* (1988) suggested that the adsorption capacity of a gelatin Sepharose column, bed dimensions 1.6 x 0.9 cm, reached a maximum after the residence time of the fibronectin in the column reached 780 seconds. Initially residence times of 375 and 417 seconds were used for this work. Decreasing the flowrate to 1.6 mL/min increased the fibronectin residence time to 938 seconds and the maximum amount of fibronectin for all the tested runs was absorbed, 98 mg. The residence time could be increased by reducing the flowrate further and a greater proportion of the fibronectin may be adsorbed. An increase in adsorption could also have been due to cleaning of the column with 8 M urea or the high loading of the column, 216 mg. Increasing the molarity of urea in the eluting buffer would also decrease the fraction of fibronectin which remains bound to the column after elution.

The yield of fibronectin from the column varied from 69-84 % with the lower yields occurring after multiple uses of the column. Thus increased efficiency may be obtained by cleaning the column with a high concentration of urea after each run. Due to the proteinaceous nature of the gelatin affinity matrix, it was important to keep microbial contamination to a minimum and reduce gelatin degradation. To assist in this all purifications were carried out at 4°C.

In order to precipitate the fibronectin from solution using PEG or to make fibronectin mats, a high concentration of the protein was required. Unfortunately only a small number of fractions from each purification run had a protein concentration greater than 2.5 mg/mL. Further examination of the variables would be required if large volumes of purified fibronectin are to be used in the process.

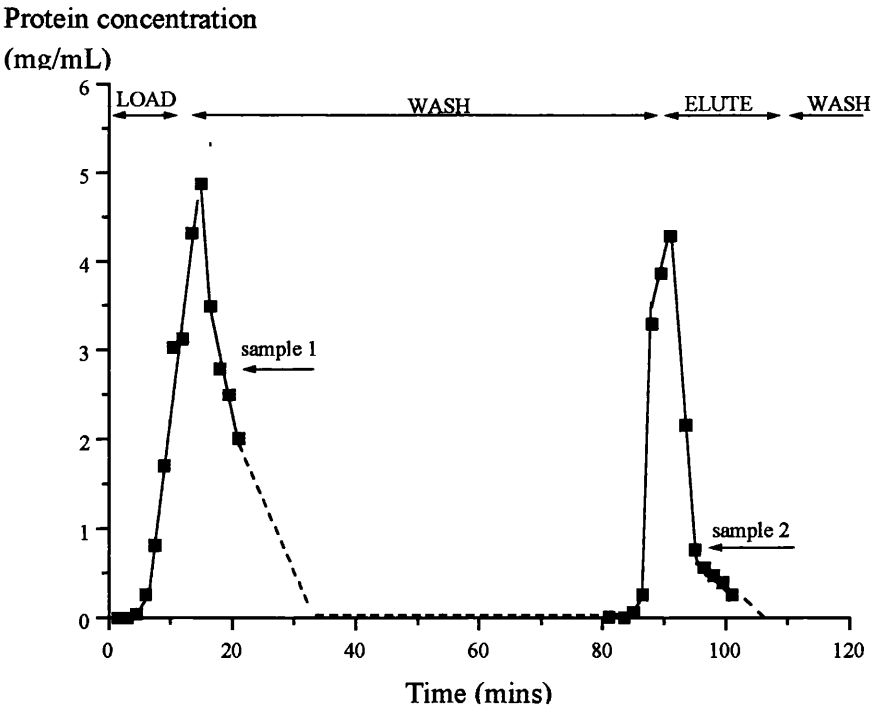


Figure 3.12. *Timecourse for affinity purification of fibronectin. A solution containing fibronectin and fibrinogen (batch 011) was loaded onto a gelatin Sepharose column at a flowrate of 3.6 mL/min. The column was then washed with 50 mM tris, 0.5 M NaCl, pH 7.6 until no more protein was collected and fibronectin was eluted with 50 mM tris, 4 M urea, 0.5 M NaCl, pH 7.6. Samples were taken as indicated and run on an electrophoretic gel, Figure 3.5. Dashed lines represent regions where fractions were not collected.*

Run Number	Total fibronectin loaded (mg) and (volume mL)	Total fibronectin adsorbed (mg)	Total fibronectin eluted (mg)	Fibronectin eluted / adsorbed (%)	Flowrate (mL/min)	Residence time (s)	Volume collected with fibronectin conc. > 2.5 mg/mL (mL)
1 (Batch 011)	128 (38)	74	62	84	3.6	417	16.2
2 (Batch 011)	74 (20)	44	35	79	4.0	375	0
3 (Batch 011)	78 (20)	56	39	69	4.0	375	0
4 (Batch 010)	216 (80)	98	79	80	1.6	938	4.8
5 (Batch 010)	221 (82)	*	82	*	1.6	938	14.4
6 (Batch 010)	97 (36)	*	64	*	1.6	938	14.4

Table 3.3. *Summary of gelatin affinity chromatography to purify fibronectin (fn). The total fibronectin adsorbed was calculated by subtracting the fn which did not bind to the column from the total loaded. The non-binding fn was calculated from the total protein breakthrough - fibrinogen loaded. Residence time was calculated for a bed height of 12.5 cm. High concentrations of fibronectin were required after column purification for fn mat making and PEG precipitation. The column was cleaned between runs 3 and 4 with 10 column vols. 0.05 M tris, 8 M urea, pH 7.5. * data not collected.*

3.5. Fibronectin mat making

Affinity chromatography purified fibronectin, derived from a glycine supernatant fraction, (BPL, Elstree) was previously concentrated and sheared in an ultrafiltration cell to produce orientated fibronectin mats. The method is described in section 2.3.1. A solution from the Protein Fractionation Centre, Edinburgh, derived from cryoprecipitate, was also purified and sheared and concentrated in an ultrafiltration cell. The mats which were produced were not highly orientated and this was attributed to the presence of fibronectin fragments in the solution which disorientated the mat. As a result the PEG fractionation step was introduced into the fibronectin production process to remove these fragments (J.Rumpus, personal communication).

A fibronectin / fibrinogen solution, which had not been affinity chromatography purified would not form mats when sheared and concentrated in an ultrafiltration cell. It had been noted previously (Ejim *et al.*, 1993) that fine fibronectin strands could be drawn upwards from the surface of fibronectin solutions which would form mats. This was regarded as a good indicator of the ability of the solution to form mats because the protein mixtures would not form such strands. Affinity chromatography of the PFC protein mixture led to a fibronectin solution in 4 M urea from which strands could be drawn. It was consequently assumed that an affinity chromatography step was essential prior to fibronectin mat formation. To determine whether it was the removal of other proteins and protein fragments from solutions by affinity chromatography which facilitated mat formation or the presence of urea in the buffer, a number of experiments were carried out to test strand formation conditions, as an indicator for a solution's potential to form mats.

Purified fibronectin solutions were in a 2 M urea, 50 mM tris, 0.2 M NaCl solution, pH 7.6 at the time of mat manufacture. The fibronectin / fibrinogen solutions were dissolved in a phosphate based buffer. To test that different buffers were not responsible for the varying behaviour of the two solutions, the buffer of the fibronectin / fibrinogen solution was exchanged for a urea containing one by dialysis. No strands could be pulled from the resulting solution. Furthermore a fibronectin / fibrinogen solution which was purified on a heparin Sepharose column (Sigma) and eluted with 4 M NaCl gave strands on

drawing from the surface, suggesting that it was the purification of the fibronectin which was important to the mat formation. The presence of urea however may also play a key role since levels of protein aggregation were reported as 2-3 times lower during mat formation in the absence of urea (Brown *et al.*, 1994). Urea acts to open out the molecule into an elongated conformation aiding fibril formation (Markovic *et al.*, 1983) as do high salt concentrations (Williams *et al.*, 1982, Tooney *et al.*, 1983). Elution with 4 M NaCl will also have produced fibronectin molecules in their extended conformation and aided strand drawing, even in the absence of urea. Thus it could be concluded that an extended conformation is critical for strand and therefore mat formation and that using a purified fibronectin solution increases the likelihood of fibronectin - fibronectin interactions required for orientated mat / strand formation. Strand pulling was enhanced in all cases when solutions were chilled to 4°C.

Having established conditions under which fibronectin mats could be made, their manufacture was examined more closely. Mats could be made from solutions with protein concentrations > 0.5 mg/mL. However thinner mats were made from lower protein concentration solutions, whilst thicker, less orientated mats were made from high concentration solutions. Fibronectin solutions could be concentrated in the ultrafiltration cell, prior to mat formation although in most cases the ultrafiltration membrane quickly became coated by a fibronectin layer, which impeded concentration to very high levels. The concentration of fibronectin and fibrinogen mixtures could be increased by approximately 65 % in the ultrafiltration cell over one hour. Figure 3.13 shows the relationship between protein concentration and flux for such a mixture. After 35 minutes the flux through the membrane has decreased 10 fold but concentration continued, suggesting that the fibronectin had formed a layer over the membrane but buffer was still being removed. Initially the flow through the membrane was 2 mL/min whereas after 55 minutes the flow had been reduced to 0.7 mL/min.

It was concluded that there were many variables involved in the production of orientated fibronectin mats, including protein concentration, impeller speed and ionic strength of the buffers and it was not deemed feasible to follow these with respect to large scale manufacture. The current technology works well as a bench-scale technique but combined with the need for large volumes of purified fibronectin, it was considered

appropriate to seek an alternative method for the large scale manufacture of fibronectin cell scaffolds.

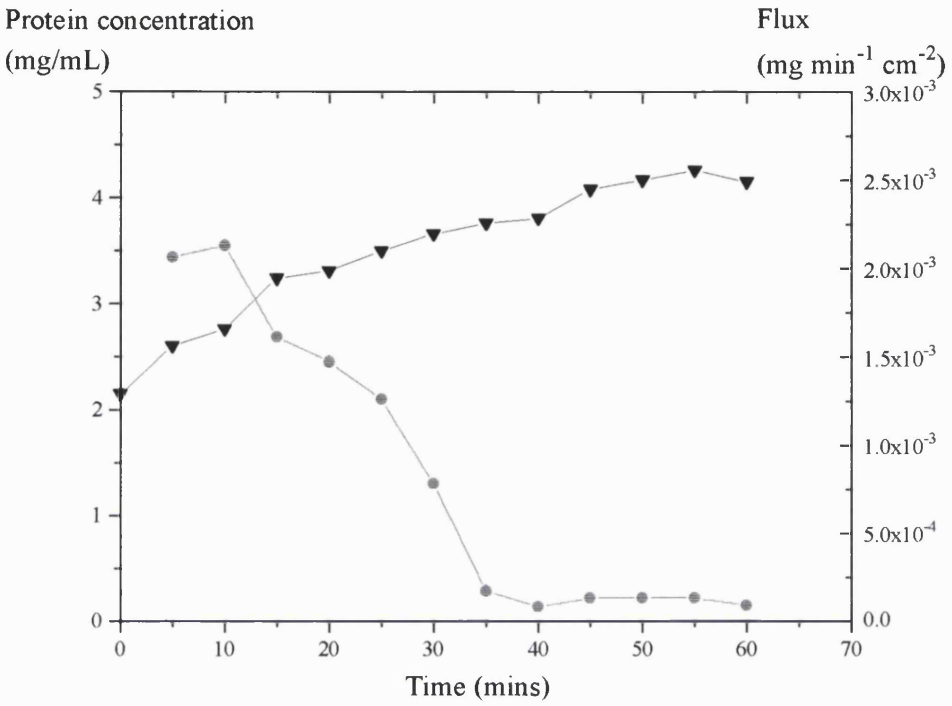


Figure 3.13. *Protein concentration, ▼, and flux through membrane, ●, for a fibronectin / fibrinogen solution concentrated in an ultrafiltration cell. The initial volume was 200 mL and this was reduced to 125 mL after 1 hour. Operational temperature and pressure were 5°C and 3 bar. The membrane used for concentration was hydrophobic with a molecular weight cut-off of 30 kDa and an area of 49 cm^2.*

3.6. Solution storage

Fibronectin was received as a sterile solution in 2 litre batches. To reduce the number of times the solution had to be thawed and refrozen for use in experimental work, the solution was thawed, split into 20 mL aliquots and refrozen. Once thawed each aliquot was kept at 4°C, until used. Due to the proteinaceous nature of the material and the possibility that sterility had been compromised during aliquoting an experiment was set up to test the effect of storage of the solution in the fridge, once the solution had been used for an experiment. Figure 3.14 shows how the turbidity of the solution, measured at a wavelength of 410 nm, increases after four days storage in the fridge. It was felt that this was related to bacterial infection and consequently degradation of the protein.

Accordingly no fibronectin / fibrinogen solution was used for an experiment after it had been defrosted, opened once, and stored in the fridge for more than four days.

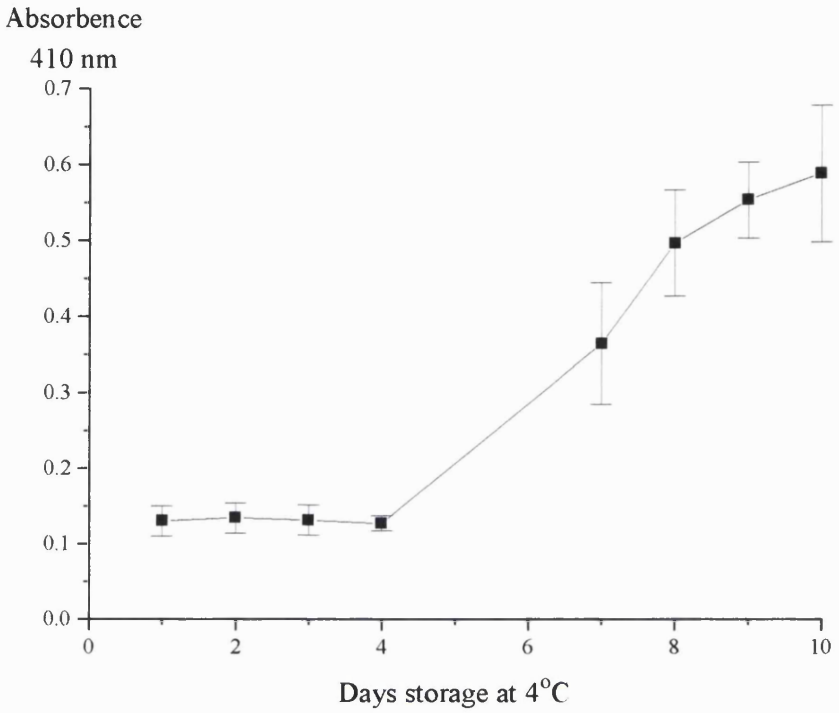


Figure 3.14. *Effect of storage time on the turbidity of a fibronectin / fibrinogen solution. Samples of fibronectin were thawed, opened once and left in the fridge at 4°C, for between 1 and 10 days. 4-6 samples were taken each day and the absorbence of the solution measured, assuming the absorbence to be proportional to solution turbidity. Results are the average of the absorbence for the samples ± 95 % confidence interval.*

3.7. Summary

The aim of this section was to develop and transfer assays so that the process of extracting fibronectin from cyroprecipitate could be monitored. It was also essential to identify key variables in the fibronectin mat making process and assess the suitability of the technique for larger scale manufacture.

Assay development: Assays for the measurement of fibronectin, fibrinogen, albumin, total protein and polyethylene glycol were transferred or developed as required. In each case it was important to choose appropriate standards for the assay and determine whether the assay was specific or whether there was some protein cross-reactivity. The

fibrinogen assay was found to be fairly specific but the fibronectin ELISA and the albumin assay showed some cross-reactivity with other proteins present and this was taken into consideration when using the assay.

Solution characterisation: The solutions were found to contain a number of protein components, including fibronectin, fibrinogen and albumin as well as residual PEG. The method for extracting fibronectin from cyroprecipitate using a 2 cut PEG fractionation was reproducible for 83 % of batches examined. The first cut removed at least 87 % of the fibrinogen and 39-76 % of the fibronectin for 5 out of the 6 batches scrutinised. The second cut removed virtually all the remaining protein to give fibronectin : fibrinogen ratios of 60:40 - 70:30. There was a failure in the first PEG cut for one batch which resulted in a high fibrinogen content in the final product. Aspects of the process, such as temperature control, may need examination to prevent a reoccurrence of this.

Fibronectin mat making: It was concluded that affinity chromatography purified fibronectin was essential for the mat formation process. This meant that immobilized gelatin chromatography would have to be performed on a large scale, increasing the cost of the process, if the current mat formation technique were to be used on a scale larger than bench-scale. Although the method performed well using a 400 mL (fibronectin volume 200mL) ultrafiltration cell and orientated mats could be made, the method was difficult to control and some fibronectin always remained in solution. These low concentration solutions proved difficult to concentrate up, due to fibronectin sticking to the concentrating membranes.

In conclusion, the knowledge gained during the solution characterisation and study of the extraction process as well as the mat making procedure will be applied to the development of alternative manufacturing strategies. These are now described in the following chapters.

4.0 Development of drawn cables and their physical properties

4.1. Introduction

Fibronectin mats and strands have been shown to provide excellent *in vivo* cell guidance/orientation properties in nerve regeneration and repair (Whitworth *et al.*, 1995a, b, 1996) and to possess properties potentially useful as depots for growth factors (Brown *et al.*, 1994) and as carriers for keratinocytes in skin defects (Prajapati *et al.*, 1996). However the methods of manufacture of these mats are not simple or amenable to reliable and reproducible production. The method described in sections 2.3.1 and 3.5 for the production of fibronectin mats is difficult to scale-up and there is a certain amount of protein wastage since not all the fibronectin in solution is converted to mat.

This chapter describes the investigation into alternative methods for making fibronectin cell guidance scaffolds. Characteristics of an ideal alternative to the mat include: a simple manufacturing method, good reproducibility and low material wastage. Cell adhesion properties must not be lost during the new method of manufacture.

4.2. Addition of acid / salt to protein solutions

A number of groups have reported the effects of pH extremes, temperature and high ionic strength environments on the molecular conformation of fibronectin (Williams *et al*, 1982; Tooney *et al.*,1983; Brown *et al*, 1987; Osterlund, 1988; Sjoberg *et al*, 1989, Benecky *et al.*, 1991). Their results are reviewed in section 1.3.3. but the main conclusions reached by the groups are summarized by Benecky *et al.* (1991). One hypothesis is that under alkaline, acidic or high ionic strength conditions the fibronectin molecule takes on an extended conformation in the form of a single extended strand, dimensions 140 x 2 nm. Alternatively it is thought that under these conditions the molecule forms 2 disk-shaped subunits (each 20 x 2.3 nm) covalently linked to one another through the C-terminus interchain disulphide bonds. Markovic *et al.* (1983b) stated that there were only small or moderate changes to the molecule's secondary

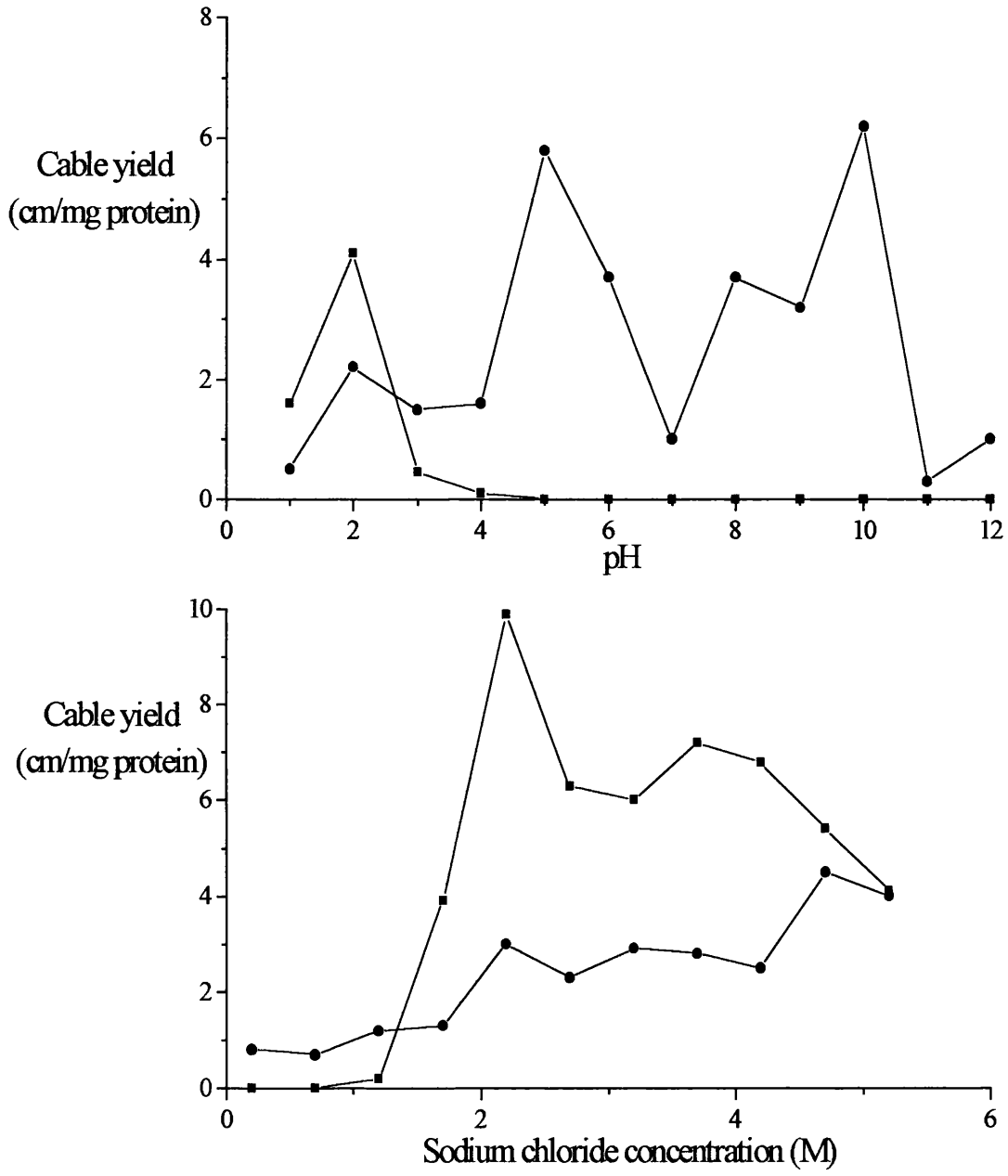
structure with extremes of pH and Khan *et al.* (1990) associated these changes with the formation and breakage of electrostatic interactions. In both cases the molecule is believed to become more flexible in 'extreme' conditions. This section reports the results of using this information to precipitate out the protein.

Initial screening experiments were performed to observe the effect of altering the solution pH and increasing the solution ionic strength using NaCl. Experiments used either affinity chromatography purified fibronectin (BPL, Elstree) or mixtures of 65 % fibronectin / 35 % fibrinogen (PFC, Edinburgh). Results for pH versus yield and ionic strength versus yield are shown in Figures 4.1 and 4.2. respectively.

For affinity chromatography purified fibronectin solutions no obvious precipitate was formed with the addition of sodium chloride up to 5 M concentrations or the alteration of pH between 1 and 12. However fine protein strands, diameter 5-10 μm, of the type described by Ejim *et al.*(1993) could be drawn for all ranges of pH and salt concentration. The length of the strands which could be drawn from the purified fibronectin solution was greatest at pH 2.0, 5.0 and 10.0. In a purified fibronectin solution all intermolecular linkages would be with other fibronectin molecules and forming linkages would be enhanced if the molecules were in an open, elongated and flexible configuration. Thus at pH 2.0 and 10.0 the molecule has a more open configuration allowing improved intermolecular interaction and improved strand drawing. The same process could be occurring in environments with increased ionic strength (Figure 4.2) causing enhanced strand drawing. Fibronectin's isoelectric point has been measured at around pH 5.2 (Osterlund, 1988) so around pH 5.0 the molecule has no overall charge and tends to aggregate leading to improved strand drawing.

The fibronectin / fibrinogen mixture formed a white precipitate when the pH was lowered to between 1.0 and 4.0 and protein cables could be drawn upwards from solution. There was no precipitate formed when the pH was increased to > 7.0 with sodium hydroxide and no cables were formed. A precipitate was also formed when the sodium chloride concentration of the solution was increased above 1 M. For salt concentrations up to 2.2 M the precipitate was fine and could be drawn upwards to form fine cables, diameter < 1 mm. However increasing the salt concentration above this led to the formation of a thick, aggregated precipitate and consequently the drawing of

much thicker cables. For sodium chloride concentrations 2.5 - 5 M it appears that the cable yield has decreased but this can be attributed to the formation of larger diameter cables.



Figures 4.1. and 4.2. *Cable yield versus pH and sodium chloride concentration for both fibronectin / fibrinogen solutions,* ■, *and for affinity chromatography purified fibronectin solutions,* ●. *Both solutions had protein concentrations 1-2 mg/mL and were mixed with an equal volume of varying concentrations of hydrochloric acid or sodium hydroxide to produce a pH range 1-12. To increase ionic strength solid sodium chloride was added to the solution to give final salt concentrations of 0.2-5.2 M. The length of protein cable which could be drawn from the solution for each set of conditions was measured and is expressed as cm cable / mg total protein in the starting solution.*

To investigate further the drawing of protein cables from solution an antibody blocking intermolecular fibronectin-fibronectin interactions was used to limit protein aggregation, Figure 4.3.

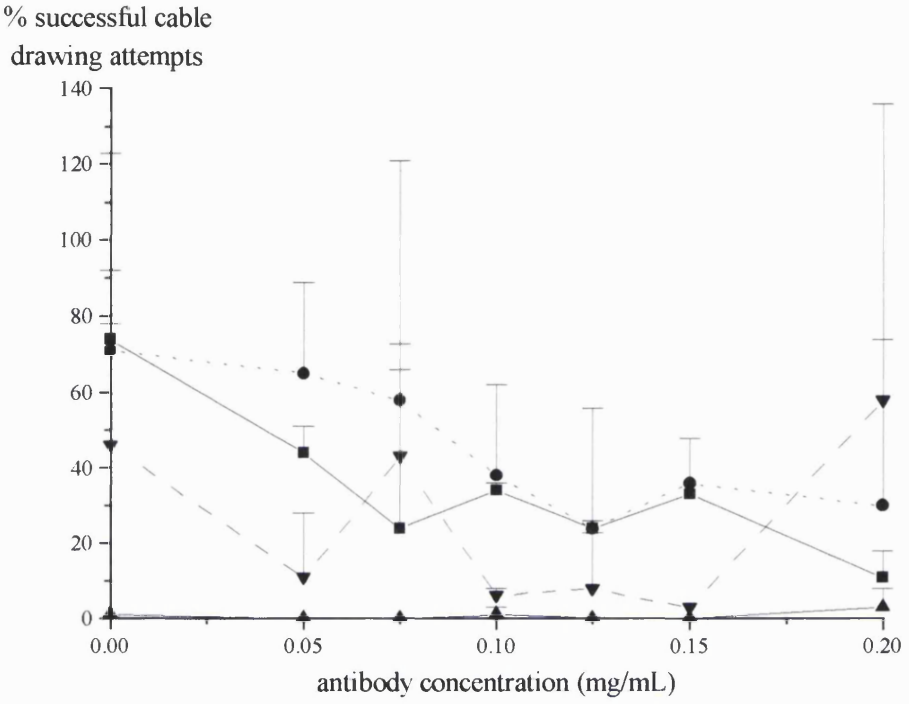


Figure 4.3. *Inhibition of cable formation by the addition of an antibody which inhibits matrix assembly by blocking fibronectin-fibronectin interactions. Antibody used was mouse-monoclonal antibody to human fibronectin, clone L8 (gift from V.Koteliansky). Antibody was incubated with fibronectin solution for 2 hours at 4°C, with or without 3 M sodium chloride. 80 attempts were made to draw up cables from each set of solution and the percentage of successful attempts recorded. Error bars represent 95 % confidence intervals for the data. Positive error bars only are shown. Purified fibronectin with 3 M NaCl - ●; purified fibronectin with no salt - ■; fibronectin / fibrinogen mixtures with 3 M salt - ▼; fibronectin / fibrinogen mixtures with no salt - ▲.*

Strands could be drawn from purified fibronectin with and without the addition of 3 M salt. However the number of successful drawing attempts increased when salt was present. The antibody at its active concentration, 0.1-0.2 mg/mL, reduced the formation of protein strands for purified fibronectin solutions both with and without 3 M salt. The non-purified fibronectin / fibrinogen mixture showed no cable formation without salt, as expected, but a precipitate was formed on the addition of 3 M salt. The position from which cables were drawn was randomised, therefore for the solution containing non-

purified fibronectin plus salt, precipitate may have been present to produce cables but the drawing attempts may have been carried out at locations where there was no aggregated protein. This would explain the low (<10 %) success rate for cables drawn for the PFC solution with 3 M salt at antibody concentrations 0.05 and 0.1-0.15 mg/ mL and the wide spread of data demonstrated by the confidence intervals. However, it does appear that cable formation from the non-purified fibronectin solution was not affected by the antibody.

It appears that strands formed from purified fibronectin solutions involve intermolecular fibronectin - fibronectin interactions whereas the presence of fibrinogen is crucial to the formation of precipitate derived cables from non-purified solutions.

Fibronectin molecules in the purified fibronectin solution will be in an extended conformation, despite neutral pH, due to the presence of urea following the affinity chromatography purification process. Markovic *et al.* (1983) report denaturation of some regions of fibronectin by urea and Khan *et al.* (1990) report the unfolding of the fibronectin molecule by another denaturing agent, guanidinium chloride.

Fibronectin - fibronectin interactions occur via either disulphide exchange at the N terminal third of the molecule, during the formation of cell associated fibronectin matrix, (McKeown-Longo and Mosher, 1989) or electrostatic interactions between the N and C termini, particularly at low temperatures (Vuento *et al.*, 1980). The fibronectin strands drawn from a purified solution are probably fibronectin molecules, in their extended conformation, linked by electrostatic interactions. *In vivo,* the plasma fibronectin concentration is only 0.3 mg/mL and in these experiments concentrations of 1-2 mg/mL were used. The increased concentration and low temperature will promote fibrillar self-assembly and peaks in strand drawing, from purified solutions, were seen under conditions when the molecules would have been in their extended configuration.

For fibronectin / fibrinogen mixtures with no added salt, only a small number of the fine fibronectin / fibronectin strands could be drawn. Fibrinogen in the solution may reduce the number of successful fibronectin - fibronectin interactions. There are a number of fibrin binding domains, one with high affinity at the N terminus of fibronectin and others with lower binding affinities towards the C terminus. The fibronectin in the non-

purified solutions is known to lack the N-terminal bacterial opsonic region (A.J.MacLeod, personal communication), closely related to the fibrin binding region. However, it is unknown whether this high affinity binding region is also lost. Fibronectin binds to fibrinogen via the C terminal end of the α chain of fibrinogen (Yamada, 1989).

It was seen that under acidic but not alkaline conditions precipitate and cables were formed from non-purified solutions, whilst only fine, 1-10 μm diameter strands could be drawn from purified solutions. Therefore we may be able to assume that there are two mechanisms responsible for strand and cable formation. Fine strands drawn up from mixed protein solutions could be formed by the same mechanism as for purified solutions, when the molecules are in an extended configuration. Large cables are formed only when the pH of mixed solutions is reduced below 4.0. or when the ionic strength of the mixed solution is increased. However such cables are not formed when pH is increased. This suggests that precipitation cannot be due solely to the formation of an extended conformation and that other precipitation mechanisms may play a role. This would also explain the reduction in successful strand drawing occurrences in the presence of the anti fibronectin - fibronectin antibody for purified solutions but the insensitivity of the mixed solutions to the antibody.

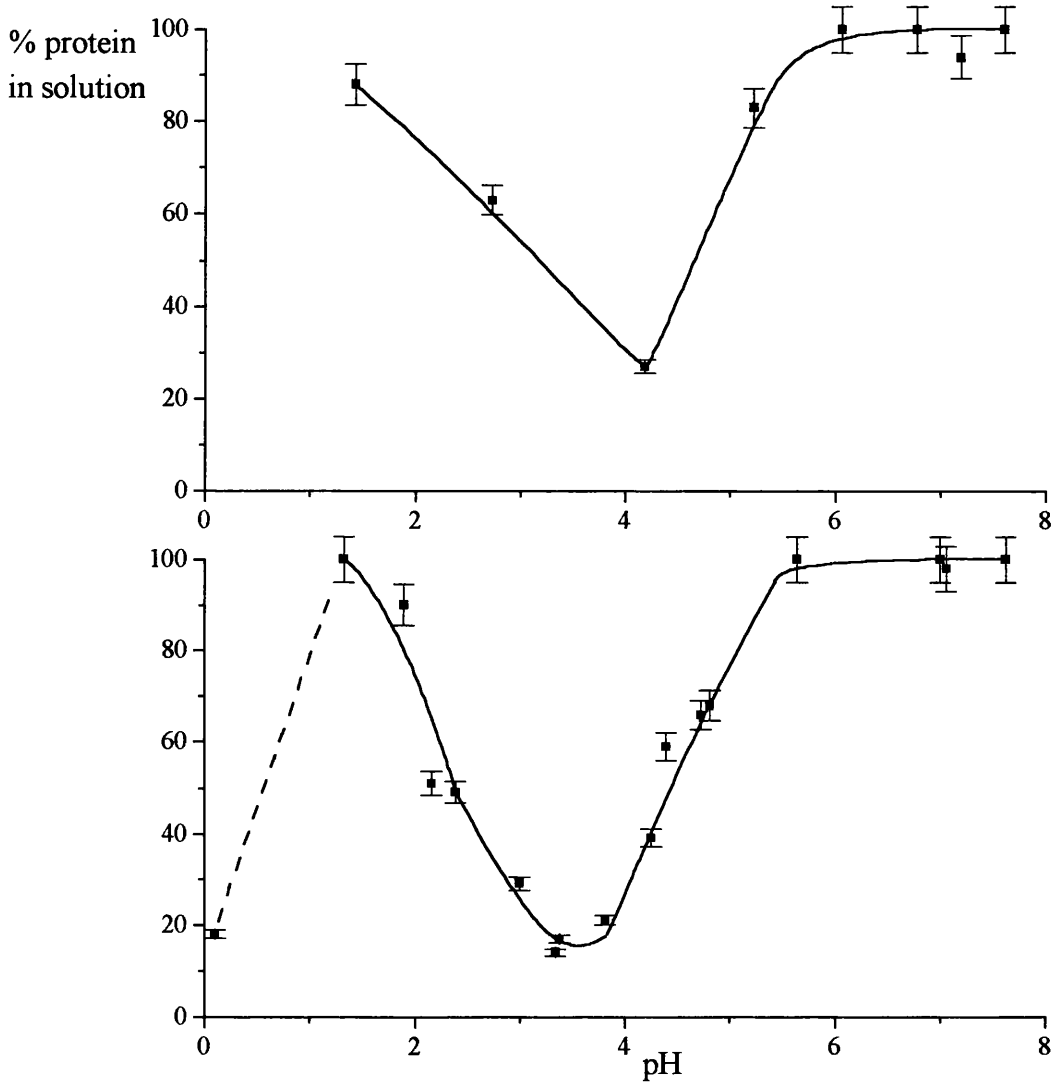
Cables drawn from high salt conditions proved difficult to stabilise and store. Washing in isotonic solutions or deionized water caused some degree of dissolution, thus further work concentrated on the more stable acid-precipitated cables.

4.3. Solubility curves

This section examines the isoelectric precipitation of fibronectin and fibrinogen and mixtures of the two proteins.

Figures 4.4 and 4.5 show pH versus solubility curves for 2 different batches of solution with fibronectin : fibrinogen ratios of 2:1 and 2.6:1 respectively. Figure 4.6 is a pH versus solubility curve for the same batch as Figure 4.5 but examining the effect of chilling to 4°C on precipitation. Figures 4.7 and 4.8 show the results of fibronectin and fibrinogen assays for these samples and Figure 4.9 is the pH versus dry weight

precipitate graph for the same experiment. Full recovery of the total volume has been assumed for all calculations.



Figures 4.4 (above) and 4.5 (below) *Solubility curves for fibronectin batches 010 and 011 respectively. Batch 010: fibronectin concentration 3.0 mg/mL and fibrinogen 1.4 mg/mL. Batch 011: fibronectin concentration 3.4 mg/mL and fibrinogen conc. 1.3 mg/mL. Error bars represent the maximum error of the mean for the total protein assay. Line represents trend in data rather than a mathematical fit (also Figures 4.6-4.12). Dashed line represents protein precipitation due to denaturation with a pH < 1.0 (Figure 4.5). pH was altered with varying concentrations of hydrochloric and citric acid at 23°C.*

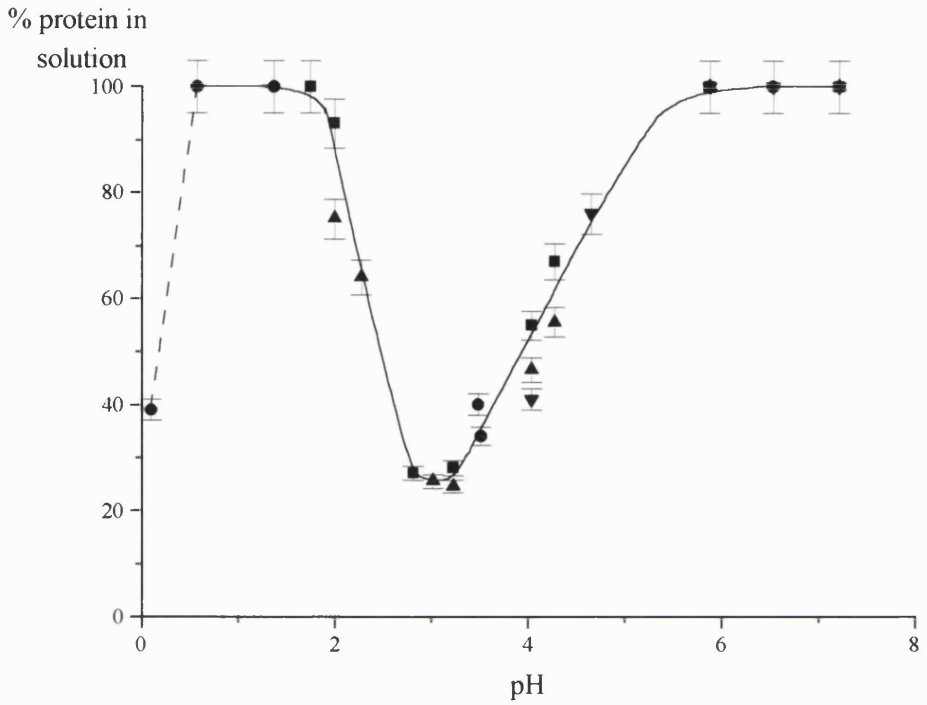


Figure 4.6. *Solubility curve for fibronectin batch 011. Citric acid (0.1 - 7.5 M) or hydrochloric acid (0.3-11 M) was used to lower solution pH and the protein remaining in solution measured using the Bradford total protein assay.* ■ *- citric acid, 23°C;* ● *- hydrochloric acid, 23°C;* ▲ *- citric acid, 4°C;* ▼ *- hydrochloric acid, 4°C.*

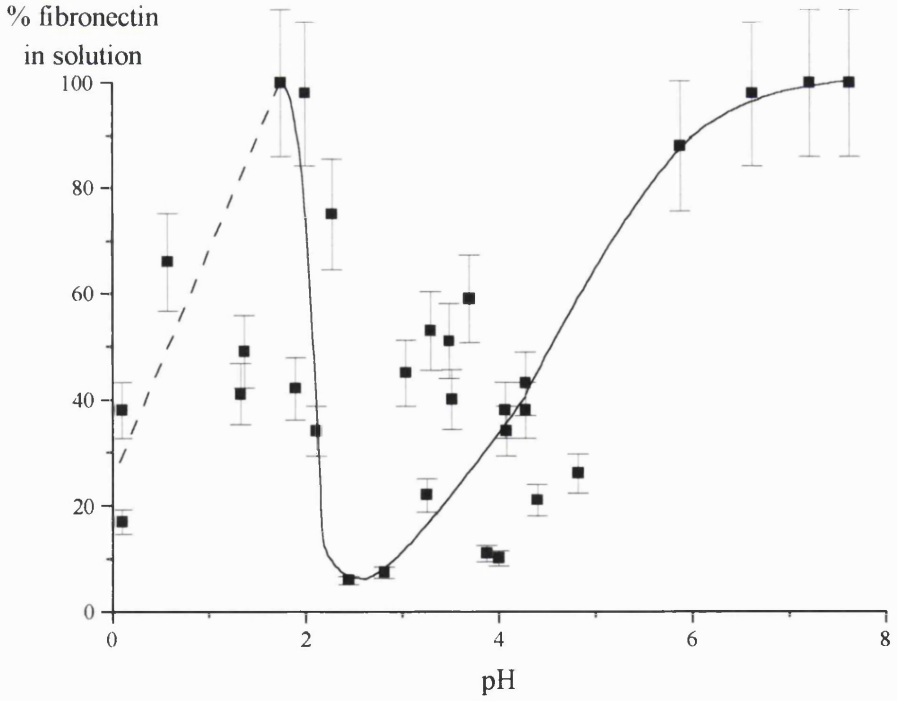


Figure 4.7. *Fibronectin remaining in solution following pH alteration, batch 011. Fibronectin concentrations were measured using the fibronectin ELISA and error bars represent the maximum error of the mean for the assay.*

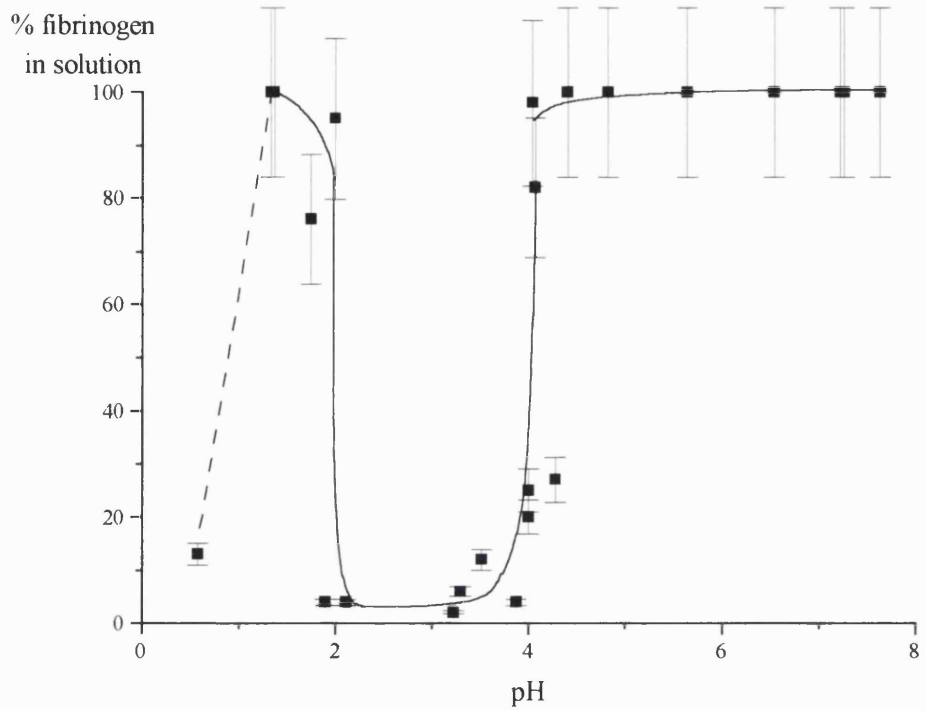


Figure 4.8. *Fibrinogen remaining in solution following reduction of pH, batch 011. Fibrinogen concentration was measured using the fibrinogen precipitation assay and error bars represent the maximum error of the mean for that assay. Dashed line represents protein precipitation due to denaturation at pH <1.0.*

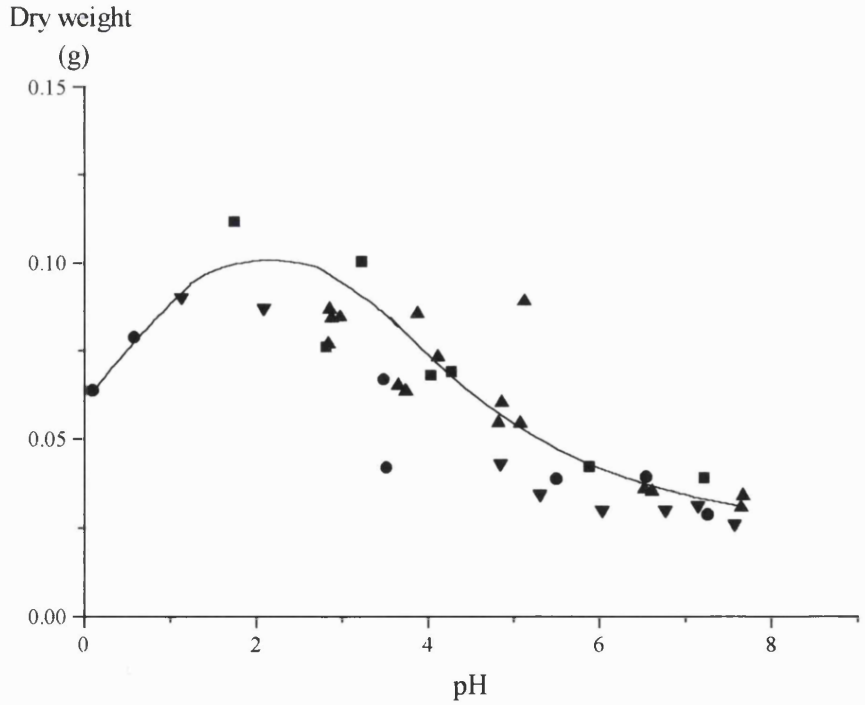


Figure 4.9. *Dry weight of protein precipitate formed for the 011 solubility curve.* ■ *- citric acid, 23°C;* ● *- hydrochloric acid, 23°C;* ▲ *- citric acid, 4°C;* ▼ *- hydrochloric acid, 4°C.*

The isoelectric point (IEP) of a protein is the pH at which the protein has no overall surface charge. Neighbouring protein molecules do not repel in their uncharged state and instead aggregate due to hydrophobic interactions causing the protein to precipitate out of solution.

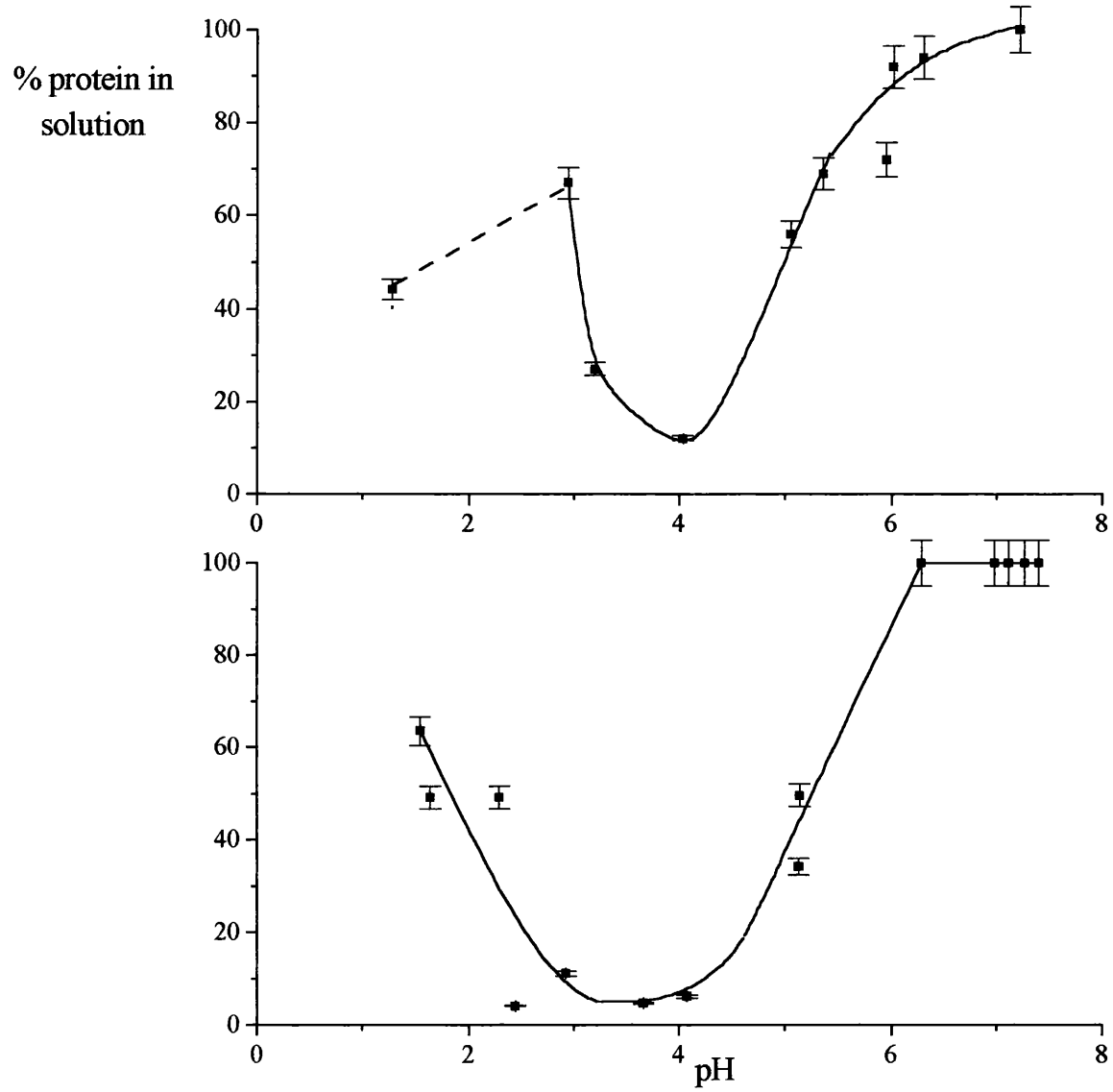
Osterlund (1988) quotes a pH of 5.2 as the isoelectric point for fibronectin, whilst values in the range 5.6 - 6.1 are given by Boughton and Simpson (1984). The IEP for fibrinogen is given as 5.5 (Doolittle, 1975). From these values the minimum point of solubility would be expected to be between pH 5.0 and 6.0. However since the solution contains a mixture of protein, heterogeneous interactions may be taking place, altering the charge-dependent protein solubilities. Experimental data shown here indicates that the proteins exhibit a minimum solubility between pH 3.0 and 4.0 and that the pH of minimum solubility is similar for batches with 2:1 and 2.6:1 fibronectin: fibrinogen ratios.

Fibronectin is a cryoprecipitative protein (Morrison *et al.*, 1948) and it may be expected that there would be a decrease in solubility at 4° C. However the shape of the solubility curve is not altered when precipitation is carried out at 4° C, Figure 4.6. Stathakis *et al.* (1978) have noted that fibronectin and fibrinogen do not precipitate, even at low temperatures, unless fibrin is present. From this it can be concluded that the mechanism of precipitation seen here is isoelectric and eliminates other possibilities such as thrombin catalysed cleavage of fibrinogen to fibrin. Altering the solution pH with mineral acids, such as citric and hydrochloric can lead to protein denaturation (Bell *et al.*, 1983). When the pH was lowered to <1.0 using concentrated hydrochloric acid, protein precipitated out of solution and aggregated. This was probably due to denaturation and is represented by a dashed line on the solubility curves.

Figures 4.7 and 4.8 show that both the major proteins in solution also follow the same solubility curve profile seen for total protein and imply that the proteins are precipitated together. Figure 4.9 shows the dry weight for total protein versus pH and again supports the conclusion that pH 3.0 is the point of minimum solubility, despite it being lower that the isoelectric point for either protein.

Figures 4.10 - 4.12 are solubility curves for solutions with different proportions of fibrinogen and fibronectin. Figure 4.10 is for a batch containing 25 % fibronectin

and 75 % fibrinogen whilst Figures 4.11 and 4.12. are for substantially pure solutions of fibrinogen and fibronectin.



Figures 4.10 (above) and 4.11(below). *Solubility curve for batch 014, fibronectin concentration 1.3 mg/mL, fibrinogen concentration 3.7 mg/mL and solubility curve for fibrinogen. The solution pH was reduced with either citric acid or hydrochloric acid and the protein remaining in solution measured using the Bradford total protein assay. Dashed line represents protein precipitation at pH< 1.0. For the fibrinogen solubility curve (4.11) freeze dried fibrinogen (PFC, Edinburgh) dissolved in phosphate buffered saline was used. Maximum concentration of fibronectin in solution was 5 %.*

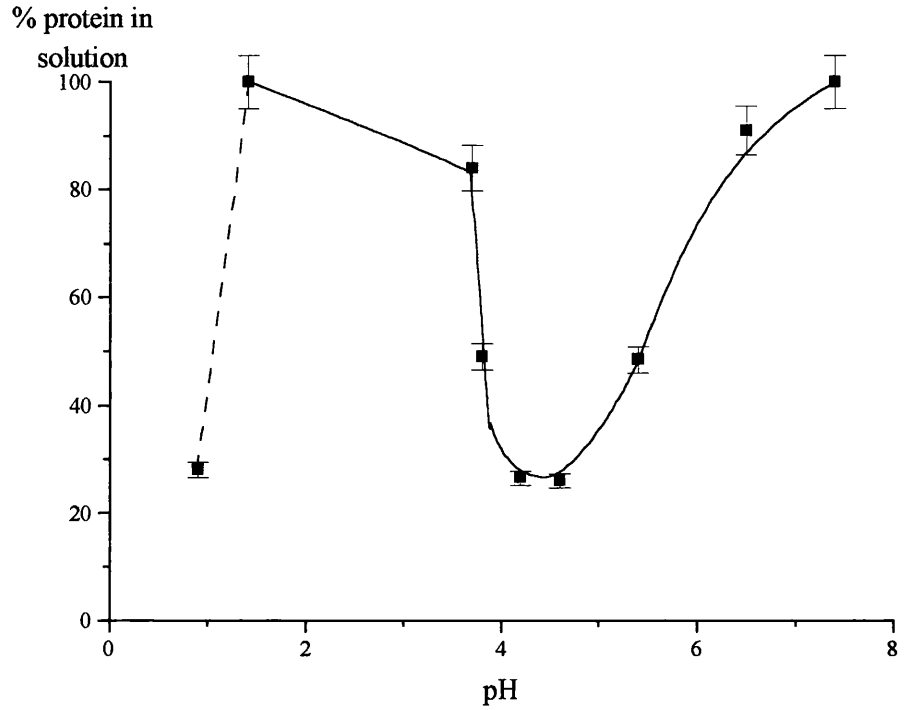


Figure 4.12 *Solubility curve for fibronectin. Fibronectin was removed from fibronectin / fibrinogen solutions by affinity chromatography, PEG precipitated and redissolved in phosphate buffered saline to increase protein concentration to 5 mg/mL. Fibronectin concentration remaining in solution was measured using the total protein assay. Dashed line represents protein precipitation due to denaturation.*

Increasing the concentration of fibrinogen in solution, Figures 4.10 and 4.11, also demonstrates a minimum protein solubility at a pH less than the expected isoelectric point. The shape of the curve is the same for the 1:3 fibronectin : fibrinogen ratio with protein denaturation at a pH around 1.0. The solubility curve for fibrinogen shows a broad pH range for its minimum solubility, between pH 2.5 and 4.0. Increasing the proportion of fibronectin in solution, Figure 4.12, where PEG-precipitated affinity chromatography purified fibronectin was used, shows a shift in the pH of minimum solubility to between pH 4.0 and 5.0. This is rising towards the expected value of 5.0 - 6.0. The low pH of minimum solubility may be caused by the fibrinogen and fibronectin molecules being forced together in such a way during processing that their charge-dependent solubilities are altered. Boughton and Simpson (1984) noticed that the isoelectric point of fibronectin in plasma was different to that in a purified solution, thus highlighting the potential for heterogeneous interactions to play an important role in a protein's isoelectric point. Markovic *et al.* (1983b) described that different domains of

the fibronectin molecule had different isoelectric points. The fibronectin used in these experiments lacked the N terminal opsonic domain, which is known to have a pI of either 6.1 (Boughton and Simpson, 1984) or 8.2 (Markovic *et al.*, 1983b). Loss of this region, combined with heterogeneous interactions with fibrinogen, may serve to lower the overall pI of the fibronectin molecule.

Markovic *et al.* (1983b) demonstrated, using sedimentation coefficient data, that the fibronectin molecule opens out into an elongated structure at a pH < 5.0. Thus both mechanisms may be occurring here - isoelectric precipitation caused by charge neutralisation, interdomain and intermolecular aggregation and increased intermolecular aggregation caused by the molecule's extended conformation. An elongation of the fibrinogen molecule at extremes of pH has also been recorded (Esouf, 1972) demonstrating an increase in the molecule's length from approximately 23 nm to 40 nm. This extended conformation should allow the C terminal fibronectin binding sites of fibrinogen greater flexibility to interact and form heterogeneous polymers.

4.3.1. pH vs viscosity

In Figure 4.13 a pH versus viscosity profile is shown for a 2.6:1.0 fibronectin / fibrinogen solution. As the pH decreased towards 3.0 the viscosity of the precipitate / supernatant mixture increased. Launay and Lisch (1979), Lee and Rha (1979) and Virkar *et al.* (1982) have all described the relationship between solution viscosity and pH during isoelectric precipitations. These groups reported an increase in viscosity of the suspended precipitate at the isoelectric point for the protein under investigation. In this study the pH of minimum solubility (pH 3.0) did not exactly correspond to the pH where the precipitate / supernatant viscosity was at its maximum, pH 1.5 to 2.5. This viscosity proved difficult to measure accurately because the formed precipitate was both fibrous and sticky and had a tendency to adhere to the walls of the concentric cylinders. Thus this discrepancy should be regarded as experimental rather than significant. Around pH 3.0 the increased cohesion between protein molecules results in a higher viscosity solution and aids the drawing of cables. An increase in viscosity was also seen at pH < 1.0, corresponding to the second pH range for protein precipitation. No conclusive trend could be drawn for the relationship of supernatant viscosity with pH. Viscosities given by the increasing shear rate and decreasing shear rate curves for supernatant were either identical or showed slightly thixotropic behaviour (average deviation 3.8 %).

However, the curves for the precipitate plus supernatant showed identical viscosities or a slight hysteresis loop representing rheopectic behaviour (average deviation 2.7 %).

There is a gradual increase in the viscosity of the supernatant and precipitate when the pH is lowered to less than 5.0. As stated previously, a decrease in pH results in the protein unfolding, an increase in the axial ratio of the protein, an increase in water taken up by the protein and thus an increase in viscosity (Lee and Rha, 1979). The viscosity of suspended particles is always greater than that of the solvent alone, due to the additional energy required to overcome solute-solute and solute-solvent interactions (Lee and Rha, 1979).

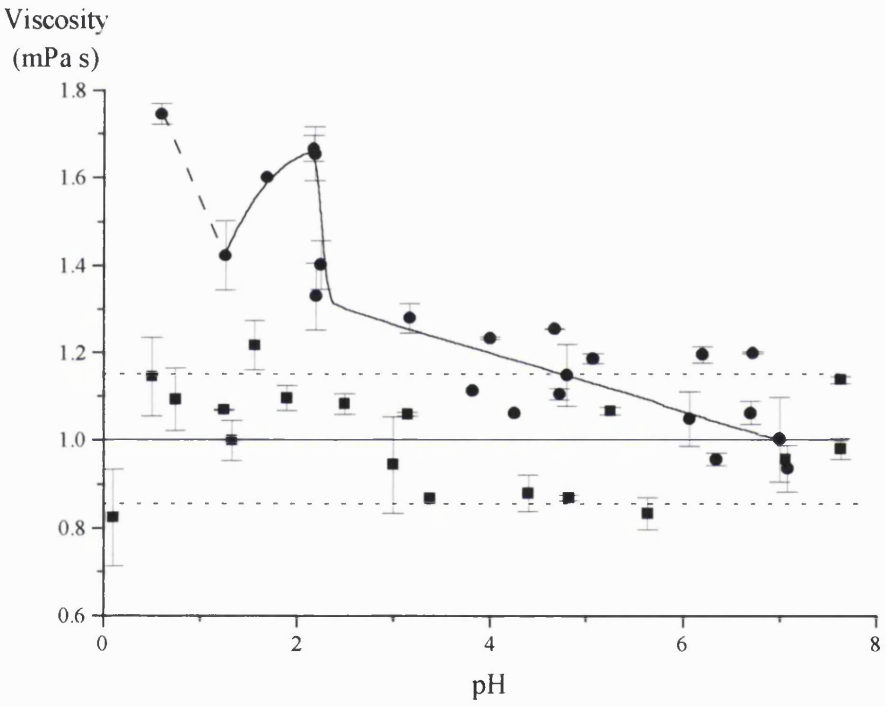


Figure 4.13 *pH versus viscosity for a fibronectin rich solution, total protein concentration 4.8 mg/mL. Viscosity of both supernatant and acid induced precipitate,* ●, *and supernatant after the removal of precipitate by centrifugation,* ■, *for shear rates between 150 - 880s^{-1}. Results are an average of the up and down curves from shear rate versus shear stress plots for each set of data. Error bars represent the range of the data where thixotropic or rheopectic behaviour was displayed. The dotted lines are the range of error calculated in section 10.2.3 for measurement of fluid viscosity in the concentric cylinders and represents the potential error in the measurement of the supernatant viscosity.*

4.3.2. Fibronectin / fibrinogen composition of precipitates

The fibronectin-rich solution used to make the cables contains fibronectin and fibrinogen in a 2:1 mol:mol ratio. The mol:mol ratio of proteins remaining in solution after cable drawing was measured and used to calculate the protein composition of the cable itself. Between pH 2.0 and 4.0, for the experiment shown in Figures 4.7 and 4.8, the protein ratio was found to range from 2:1, representing the ratio in the starting solution, to 1:1, when less fibronectin was precipitated. However cables made from other batches of PEG precipitated protein had fibronectin:fibrinogen molar ratios of 3:2 to 5:2 and these changes were reflected in the composition of the cables.

During wound repair both fibrin and fibronectin are major components of the haemostatic plug. Fibronectin is important for cell adhesion and fibrin is the major protein in the clot material. Traditionally, purified fibronectin has been used as a coating material for *in vitro* cell adhesion experiments rather than combinations of the two proteins. It should be noted that while it is vital that cells adhere to the substratum it is also essential that cells will migrate along a surface in order for tissue repair to occur. Corbett *et al.* (1996) have described plasma fibronectin cross-linked to fibrin in a complex multimer as being functionally distinct to fibronectin coating a plastic surface. They found that NIH-3T3 cells neither attached nor spread on cross-linked fibrin alone but achieved 50 % of maximal cell area seen on purified fibronectin when a fibronectin-fibrin matrix was used. This work supports results seen by Grinnell *et al.* (1980) where they found that BHK fibroblasts did not bind to fibrinogen or fibrin alone but bound in the presence of fibronectin. This cell adhesion could then by increased by covalently cross-linking the fibrin to fibronectin. In contrast, Donaldson and Mahan (1983) examined the closure of skin wounds in adult newt hind limbs when either fibrinogen or fibrin coated coverslips were implanted into the wound. They found that epidermal cell migration did not differ between either the fibrinogen coated coverslip or the fibronectin coated samples. Both samples showed similar migration rates to the epidermal wound bed.

Cells use fibronectin to interact with extracellular matrix protein such as collagen via the cell and collagen binding domains on the fibronectin molecule. A complex of proteins which spans the cell plasma membrane, called integrin, binds to the cell adhesion

sequences in the fibronectin molecule. The fibronectin may also be bound to collagen allowing the cells to use collagen as a network for their growth and movement. Based on this, the possibility exists to use just fibronectin as a guide for cell movement. However, recent studies of cell interactions with fibronectin by Palecek *et al.* (1997) involved studying levels of integrin and ligand, their interactions and binding affinities. A reduction in cell migration speed was found when a concentration of fibronectin greater than optimal was used. Although cell adhesion is improved with high concentrations of fibronectin, the protein itself may be too 'sticky' to allow the cells to detach rapidly, as is required for migration. Addition of a second component, such as fibrinogen, to the material may serve to increase the rate of cell migration along the material without hindering cell adhesion.

Studies of cable composition revealed that the fibronectin : fibrinogen ratio in the cables approximated that in the starting solution. It may therefore be possible to alter the cable's composition by altering the protein ratio in the starting solution. Ahmed *et al.* (1998c) have demonstrated that dermal fibroblasts and Schwann cells migrate at different speeds along cables with varying protein compositions with ratios of 50 % fibronectin : 50 % fibrinogen promoting the fastest cell migration rates.

4.4. Optimisation of precipitation

It was found that addition of hydrochloric acid to the solution resulted in the formation of a highly aggregated protein precipitate, which was difficult to draw. However the addition of the weaker citric acid resulted in the production of a white fibrous precipitate which could be drawn away from solution to form protein cables. This section describes the optimisation of a process to produce these drawn cables.

4.4.1. Acid and protein concentration

Optimisation experiments centred around the choice of appropriate initial protein concentration and citric acid concentration. Figure 4.14 shows results of protein precipitation from solution with the addition of 0.01 - 1.0 M citric acid for solutions with protein concentration 0.4 - 3.5 mg/mL.

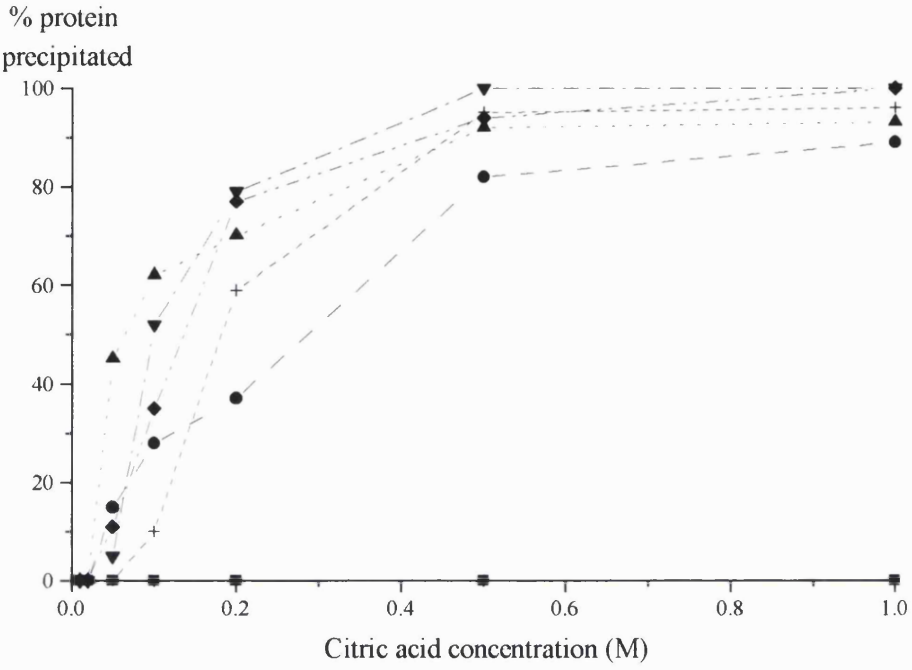


Figure 4.14 *Measurement of protein precipitation for a range of citric acid concentrations and protein concentrations. Fibronectin solutions were diluted with citric acid concentrations, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0 M to give final protein concentrations:* ■ *- 0.43 mg/mL;* ● *- 1.57 mg/mL;* ▲ *- 2.35 mg/mL;* ▼ *- 3.13 mg/mL;* ◆ *- 3.53 mg/mL;* + *- 3.92 mg/mL and the amount of protein precipitate formed was measured using a dry weight method.*

Increasing the concentration of citric acid was found to increase the amount of protein precipitated from solution for all protein concentrations except 0.4 mg/mL. No protein could be precipitated from this dilute solution even with the highest citric acid concentration tested, 1.0 M. Citric acid concentrations 0.5 and 1.0 M formed precipitates which were difficult to draw into cables whilst lower citric acid concentrations, < 0.1 M, did not precipitate much protein (<20 % for all dilutions except 2.35 mg/mL). It was concluded from this that either 0.1 M or 0.2 M citric acid should be used. Dilution of the solution to 1.6 mg/mL with acid did not produce as much precipitate as more concentrated solutions. 0.1 M citric acid was chosen to perform the remaining optimisation experiments in an attempt to reduce the concentration of acid in the system.

0.1 M citric acid was used to produce a titration curve with the fibronectin solution, Figure 4.15 It was found that when enough acid was added to reduce the pH to below 5.0, at a protein concentration of 3.6 mg/mL, precipitate was formed. This supports the solubility curves (Figures 4.4, 4.5) which show protein precipitation with pH < 5.0.

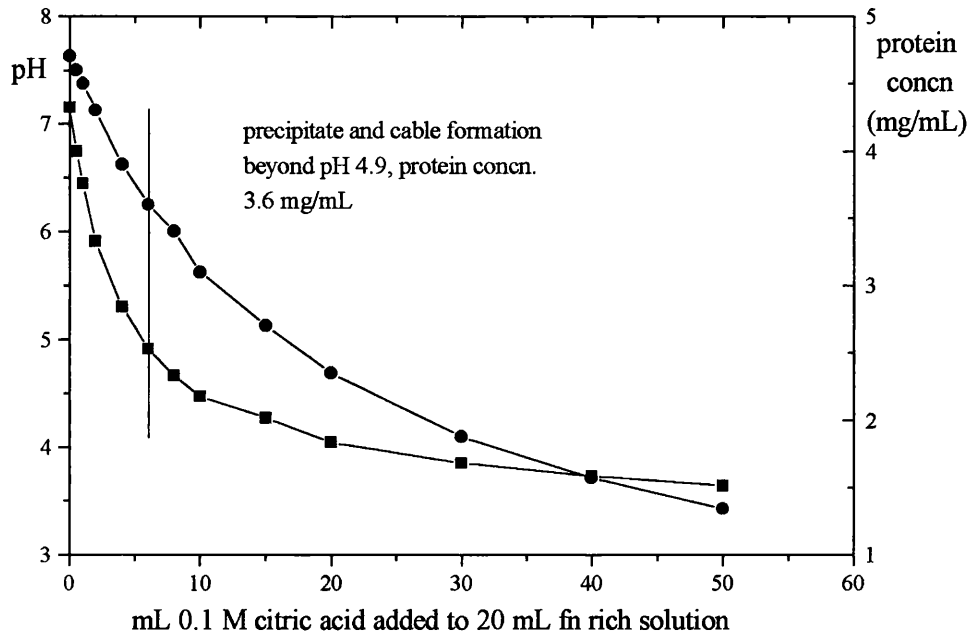


Figure 4.15. *Titration curve for 0.1 M citric acid and fibronectin solution, total protein concentration 4.8 mg/mL. Total protein concentration following dilution by acid (precipitated and non-precipitated) - ●; pH - ■.*

Figure 4.16 gives results for different acid addition strategies and temperature considerations. Maximum protein precipitation occurred when the protein concentration was diluted to between 2.5 and 3.75 mg/mL with acid. The amount of protein precipitated was not dependent on the order of acid or fibronectin addition. Chilling to 4°C also did not enhance precipitation. The proportion of protein precipitated was slightly higher when the experiment was scaled up from 1 mL to 5 mL. This could be due to precipitated protein drawing more protein out of solution.

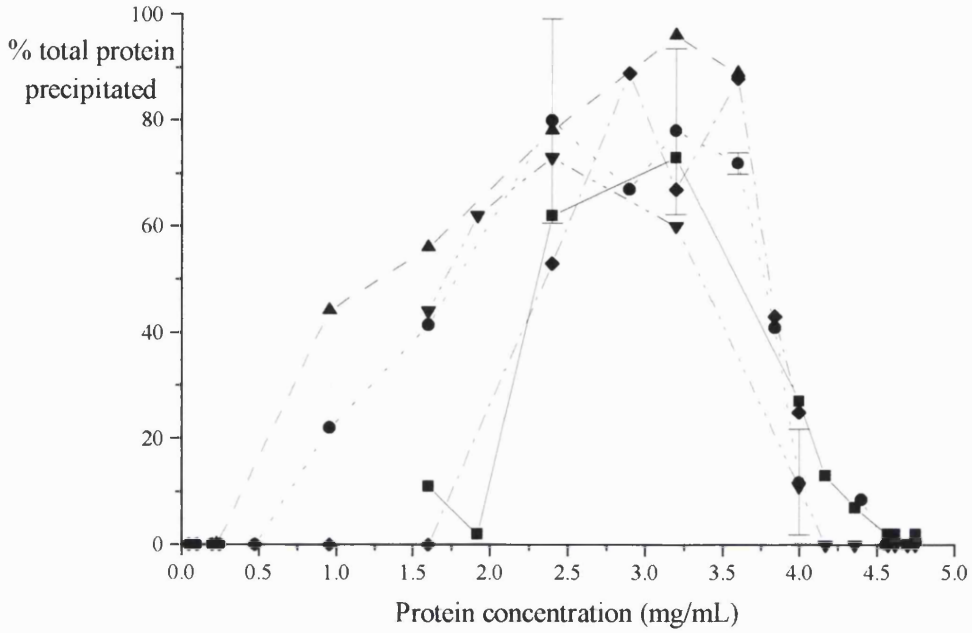


Figure 4.16. *Protein precipitation for solutions diluted with 0.1 M citric acid, pH 3.5. Acid was added to 4.8 mg/mL fibronectin-rich solutions to give a range of protein concentrations.* ■ *- 1 mL fibronectin solution with varying volumes of acid. 23°C;* ● *- 1 mL acid with varying volumes of fibronectin, 23°C;* ▲ *- 5mL acid with varying volumes of fibronectin, 23°C;* ▼ *- 1mL fibronectin solution with varying volumes of acid. 4°C;* ◆ *- 1 mL acid with varying volumes of fibronectin, 4°C. Protein precipitation was measured using a dry weight method. Error bars represent 95 % confidence intervals where experiments were repeated.*

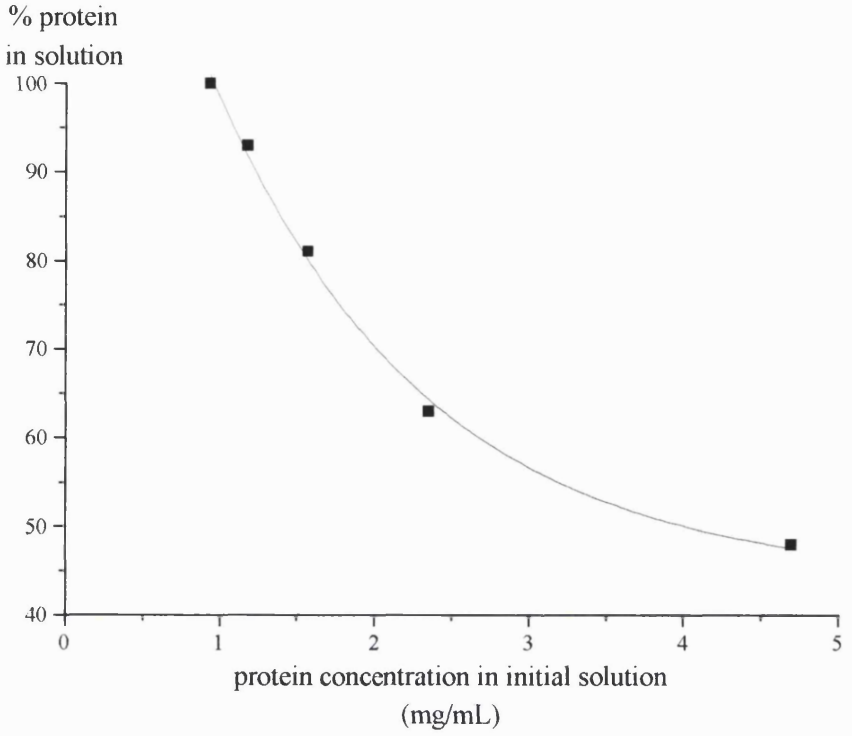


Figure 4.17 *Total protein concentration in the initial solution versus protein precipitation. 0.1 M citric acid, pH 3.4 was added in the ratio of 1 vol. acid to 2 vols. protein solution to a range of protein solutions pre-diluted with phosphate buffer. Amount of protein precipitated was measured using a dry weight method.*

Figure 4.17 shows that protein cannot be easily be precipitated from solutions with concentrations less than 1 mg/mL.

The final conditions decided upon were 0.1 M citric acid to dilute a solution with total protein concentration 4.8 mg/mL, 65 % fibronectin and 35 % fibrinogen, to a concentration of 3.2 mg/mL. That is 2 volumes of protein solution were added to 1 volume of 0.1 M citric acid. This resulted in a solution with pH 4.0 - 4.5. From the solubility curve (Figure 4.5), 40-60 % of the total protein remained in solution at this pH. However after drawing cables at this pH the final protein concentration in solution was only 20 % indicating that once cable drawing started, more protein was precipitated from solution. Cables had to be drawn upwards from solution within ten minutes of acid addition to the protein otherwise the precipitate began to adhere to the dish.

4.5. Physical properties

Cables were made as described in section 2.3.2. and their physical properties examined to assess their suitability for large scale manufacture.

4.5.1. Yield measurements and dimensions

The precipitation process was found to yield the equivalent of at least 8 cm cable / mg soluble protein with cable lengths usually drawn between 2 and 20 cm. Operational experience at PFC found that approximately 10 g cryoprecipitate could be retrieved from 1 litre of human plasma donation and that the fibronectin and fibrinogen yield from cyroprecipitate was 2.8 % (g protein / weight cryo.). Consequently, in theory at least 13 metres of cable could be produced from 1 litre of plasma.

The denier or fineness of the cables was 65 ± 6 %, which corresponds approximately to a diameter of 200 μm. Using scanning electron microscopy the cables were seen to be made up of numerous finer filaments, diameter 1 μm (Figure 4.18). These measurements were confirmed using the image analysis system to measure toludine blue stained cables.

The micron-diameter fibrils which constitute the main cable could act as a guide for cell migration. Abercrombie (1980) described the orientation of cells along a glass rod of diameter 100 μm but demonstrated no orientation on a rod of diameter 245 μm. This is attributed to cells sensing the curvature of the material by the relative position of parts of their cytoskeleton. The threshold diameter for orientation of cells on a glass rod was given as 200 μm. den Braber *et al.* (1996) used various microgrooved surfaces to demonstrate cell orientation of rat dermal fibroblasts and they found that if ridges on their substratum were greater than 4 μm wide the cellular orientation was random ($> 45^{\circ}$ from parallel to the ridge). However if the ridge was less than 4 μm wide then the cells were highly orientated ($< 10^{\circ}$).

The drawn fibronectin cables are approximately 200 μm in diameter with a surface textured with micron-diameter ridges. From the work described above the topography

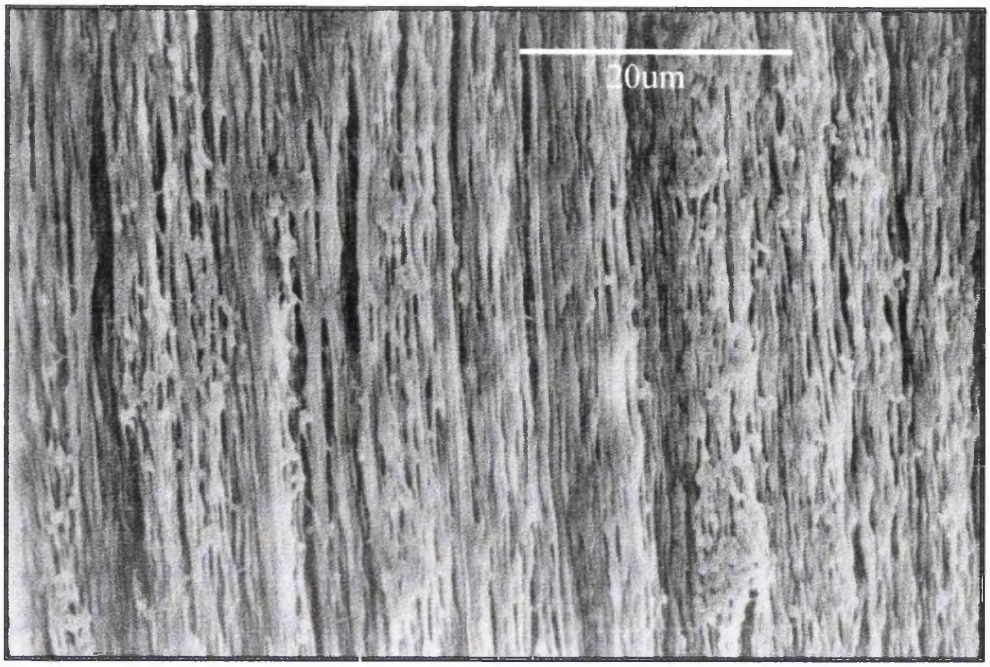


Figure 4.18. *Scanning electron micrograph of the surface of a drawn fibronectin cable demonstrating the parallel micron-diameter fibrils which give the material ultrastructural orientation.*

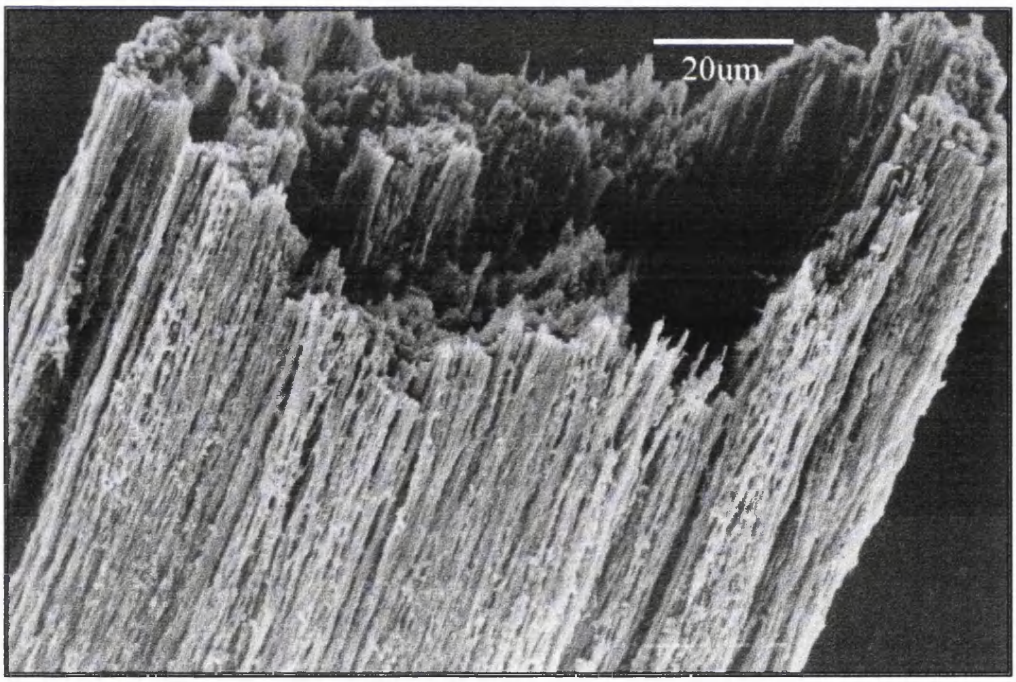


Figure 4.19. *Scanning electron micrograph showing the end of a non-twisted, drawn cable. Parallel micron-diameter fibrils are visible on the surface. The view of the end of the cable demonstrates a non-compacted centre.*

of the surface ridges should act as a form of 'micro-guidance', aligning each cell. The combination of this with the cell adhesion properties of fibronectin and the fact that cells appear to use fibronectin *in vivo* as an aid for guided migration is anticipated to promote the use of these fibronectin materials as contact guidance substrates. Results of the interaction of cables with cells have been reported for dermal cells by Harding *et al.* (1998) with a view to skin replacement and for Schwann cells by Ahmed *et al.* (1998d) with a view to nerve repair.

4.5.2. Tensile strength measurement

All tissues have to withstand both stresses and strains *in vivo*. However, the main load bearing protein in the body is collagen, not fibronectin. Collagenous fibres are responsible for providing resistance to force, tension, stretch and pressure whilst providing structural support and elasticity in organs and tissues such as skin, bone, tendon, ligaments, cartilage, connective tissue, smooth muscle and blood vessels (Ross *et al.*, 1989). In comparison, fibronectin is primarily a cell-adhesion protein and is not expected to exhibit the high tensile properties shown by collagen.

During tensile testing of moist specimens it was found that the application of a constant strain rate, 2mm / min, at a constant relative humidity (65 %) resulted in extrusion of the material. At this strain rate the cable length increased up to 7-fold with a consequent decrease in diameter. Thus the cables may be drawn down to the required diameter once they are formed, provided that the material moisture content is at least 30 % and the air relative humidity is at least 65 %. Results for air dried specimens are given in Table 4.1.

Based on standard textile processing, the cables were twisted as they were drawn from solution. In textile industries this is done to increase the elongation at break and the tensile strength up to a certain point. Over twisting causes the material to deform and weakens it (Moncrieff, 1969; Booth, 1983).

Material	n	Maximum tensile strength N/mm^2 ($\pm$ C.I)	Elongation at break % ($\pm$C.I)
Bulk	2	22.5 ± 6.9	2.7*
Non-twisted cables	2	14.9 ± 3.5	4.9 ± 4.1
Twisted cables	11	60.9 ± 8.5	3.5 ± 0.4
Twisted cables with identified defects	10	22.9 ± 5.1	3.1 ± 1.7

Table 4.1. *The maximum tensile strength and elongation at break of air dried fibronectin / fibrinogen conjugate cables. Bulk material is the protein precipitate which has been moulded into a fibre rather than drawn. Elongation at break is the increase in sample length after testing expressed as a percentage of the initial length. n represents the number of samples tested. Confidence intervals (C.I.) are at the 95 % level.* * *length of only one sample measured. Measurements were taken at 23° C, 40-50 % relative humidity. Variation of tensile strength within specimens was found to be < 10 %.*

Twisting the fibronectin material while it was being drawn led to an increase in cable maximum tensile strength due to the presentation of previously remote binding sites to each other and thus cross-linking within the cable. Twisting the cables also removed water held within the structure and may have enhanced the formation of hydrophobic interactions. Figures 4.19 and 4.20 show cross-sections of cables which have been drawn straight from solution or twisted after drawing from solution respectively. The non-twisted cable has a relatively open core, whilst the twisted cable has a compacted core leading to the increased tensile strength associated with twisted cables, 61 N/mm^2. It should be noted that twisting must occur immediately after cable formation before the material loses its adhesive properties. Defects in the cables were observed when twisting was carried out after the material had lost its self-adherent properties. The final product was a cable containing cavities where air had been trapped and a low tensile strength, Figure 4.21. Cables without defects appeared glassy whilst those with defects were opaque and air bubbles could be viewed under the light microscope.

Engineering materials such as nylon 66, teflon and PVC have maximum tensile strengths of 62 - 83, 10 - 12 and 48 N/mm^2 respectively (RS Components Ltd, Corby, UK). Values for nylon and PVC are similar to that of the fibronectin cables whilst the fibronectin is at least 5 times stronger than teflon in its non-flawed form. Unfortunately

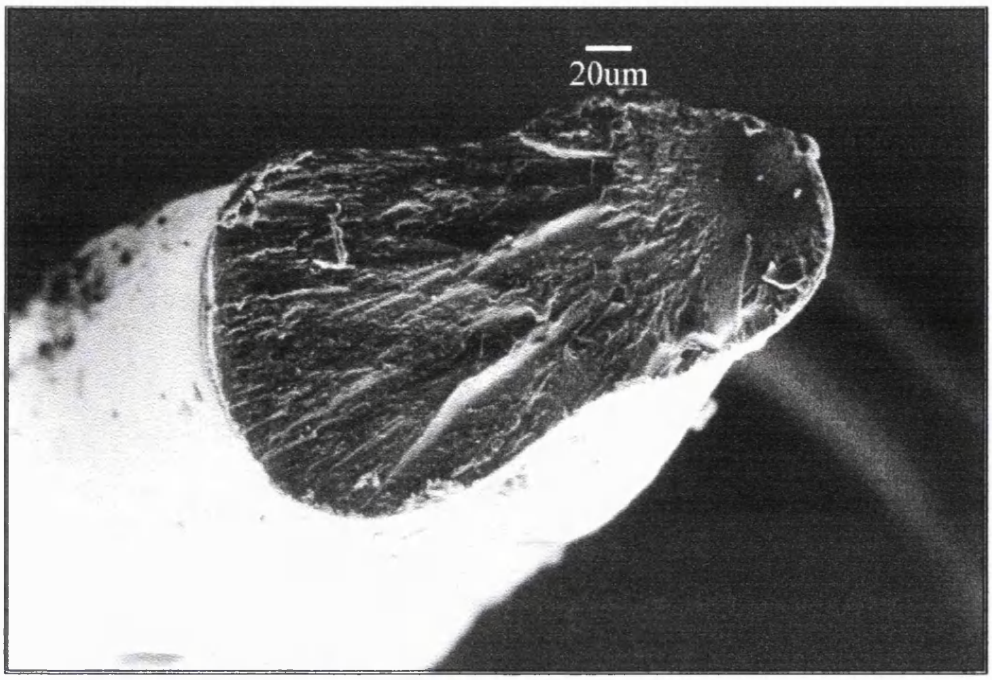


Figure 4.20. *Scanning electron micrograph of a cable which was twisted as it was drawn out of solution. The end of the cable demonstrates a compacted centre, which leads to the increased tensile strength seen for the twisted cables. The smooth end has the appearance of typical fracture site for a brittle material and the cross-sectional area is more oval than that seen for the non-twisted cable.*

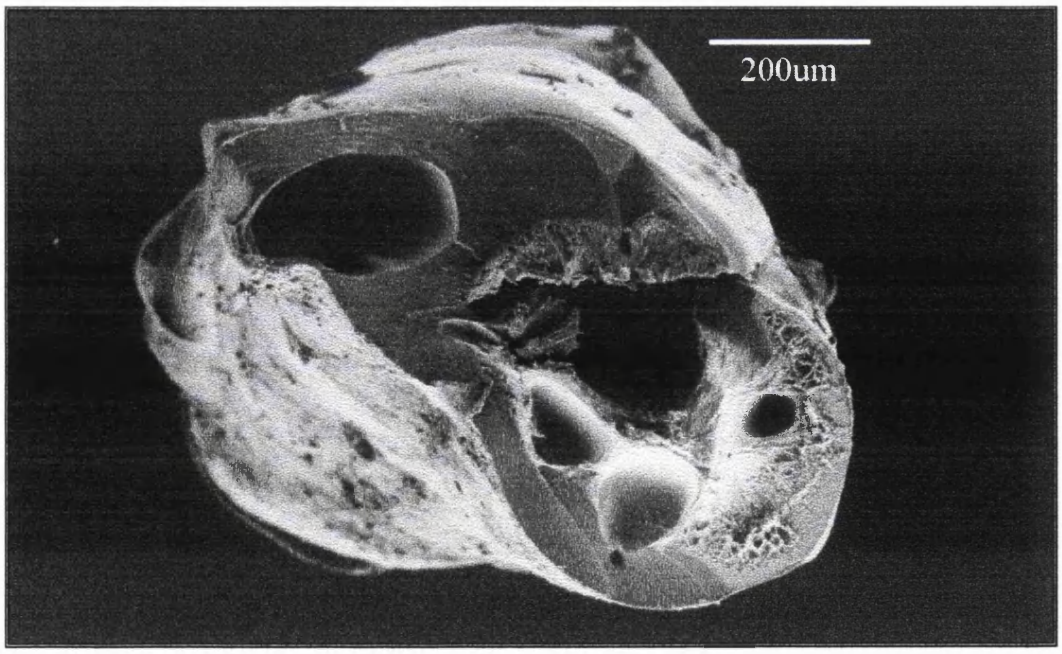


Figure 4.21. *Scanning electron micrograph of a cable which was twisted during manufacture but where the component fibrils did not adhere well. The result was a large cable containing numerous air-filled cavities and a low tensile strength.*

characteristics of the stress / strain plot (Figure 4.22) and the fracture site identify the material as highly brittle and thus difficult to manipulate.

The elongation at break is a good measure of the 'workability' of a fibre in textile processes such as weaving and knitting. Usually a minimum elongation at break of around 10 % is required to ensure textile working (Moncrieff, 1969). The elongation at break for fibronectin cables, 3 - 4 %, is typical of a dry, brittle material and indicates a material which is difficult to handle.

Tenacity is another measurement of fibre strength often quoted in textile literature. Tenacity is defined as the specific strength of the material and is based upon the gram weight required to fracture the specimen per denier of the fibre (where denier is the linear density of the material). The tenacity of fibronectin / fibrinogen fibres is compared to that for other fibres in Table 4.2.

Fibre type	Tenacity (g/denier)
Fibronectin / Fibrinogen cables - twisted (dry)	2.50 - 3.50
Fibronectin / Fibrinogen cables - non twisted (dry)	0.73
Soybean (dry)	0.80
(wet)	0.25
Casein (dry)	0.80 - 1.00
Vicara (maize) (dry)	1.20
Polyglycolic acid (dry)	4.50- 5.30

Table 4.2. *Tenacity of a number of protein fibres (Moncrieff, 1969) compared to fibronectin cables (Underwood et al., 1999) and a synthetic fibre, polyglycolic acid, (Freed et al., 1994) currently being developed for use as a scaffold for chondrocytes. The denier before tensile testing was used to calculate tenacity and no correction was made for the material being drawn to a finer diameter during testing.*

Results for wet spun fibronectin materials are compared in section 6.3.1.

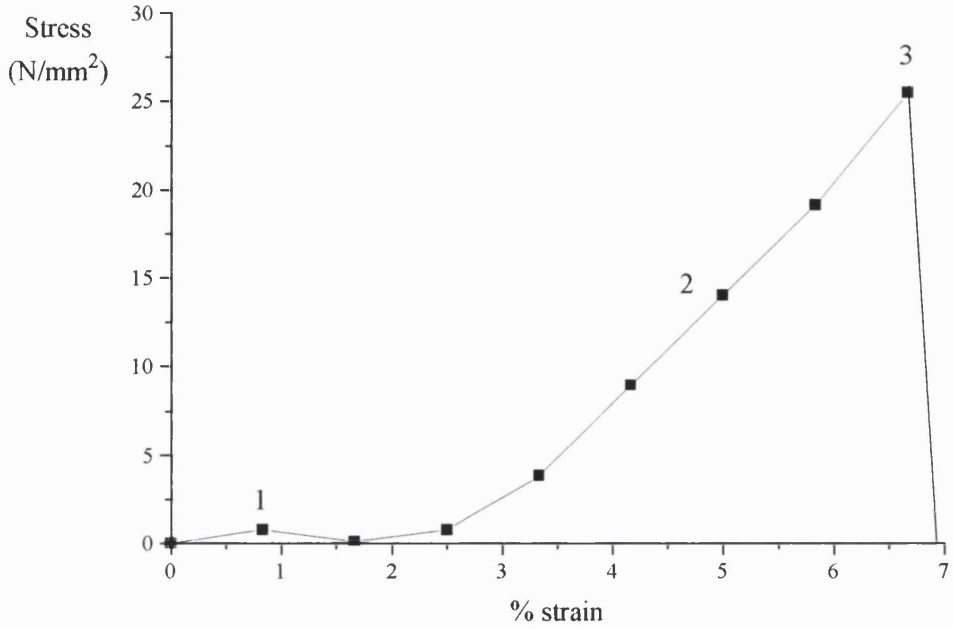


Figure 4.22. *Typical stress / strain curve for a twisted, drawn fibronectin cable. This example was one of the twisted cables which was found to have been twisted after it had lost its self-adhesive properties. Stress is calculated as load applied per unit area and expressed in N/mm² (Cable area = 0.2 mm²) . Strain is expressed as an increase in the original length of the cable. Here the cable has ultimate tensile strength 25 N/mm².*

The regions of the profile can be explained as follows:

1 - the material extends to its full length, i.e.: removing the slack from the system.

2 - the material behaves elastically, and obeys Hookes Law where stress is always proportional to strain

3 - material fails at its maximum or ultimate tensile strength. The actual value is calculated by dividing the load required to induce failure by the cross sectional area of the failed material. This is typical of a brittle material, in that it fails under loading at a relatively low strain (Gere and Timoschenko, 1991).

4.5.3. Solubility

For this material to act as a template for tissue regeneration, it must remain in the body long enough for new tissue to repair the damaged area. However it is also essential that the material can eventually be broken down and eliminated from the wound site. During processing it is necessary to wash the material without disintegration so that

chemical residues which are not desired in the final product can be removed. In order to remove the citric acid present in the cables following preparation, they were washed in deionized water or phosphate - buffered saline (0.01 M) immediately after formation. However this resulted in some disintegration of the material and it was important to develop a non-chemical means of stabilisation. Severe desiccation has been reported to stabilise aggregated protein materials such as collagen, (Yannas, 1995) so air drying, freeze drying and oven drying at 37°C were used to dehydrate the material and increase the intermolecular bonding. Comparative results are given in Figure 4.23.

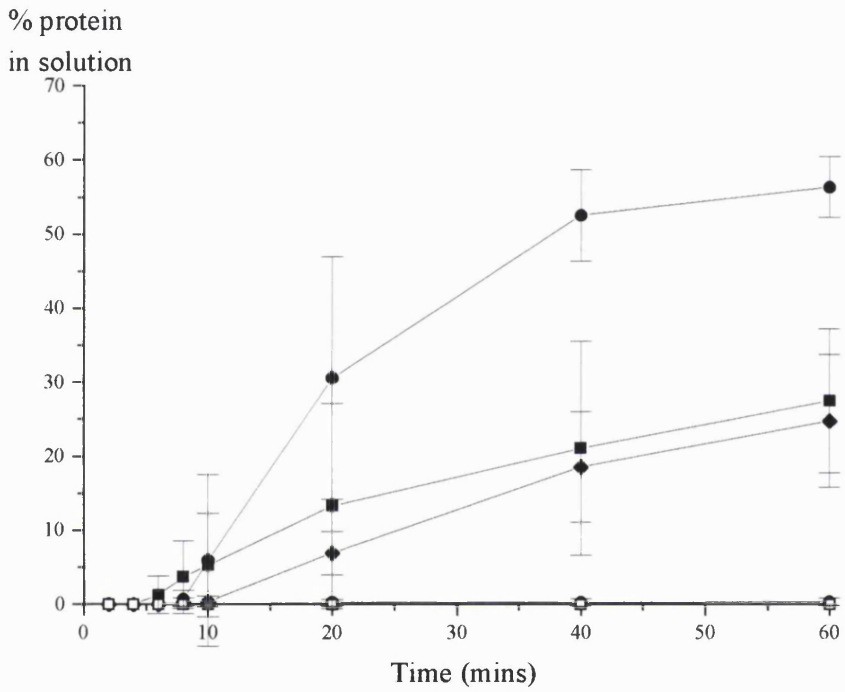


Figure 4.23. *Methods of stabilisation of fibronectin material during a potential water washing stage of material at room temperature.* ● *- freshly prepared material;* ■ *- freeze dried material;* ◆ *- 1 hour air dried material;* ○ *- 24 hours air dried material;* □ *- 24 hours dried at 37°C. Data shown are the average of three experiments and error bars represent the 95 % confidence interval for the data*

Air drying the material for 24 hours and drying in an oven at 37°C resulted in no protein being lost from the aggregates of material over a 60 minute period. Freeze drying the material increased the material's porosity and thus susceptibility to dissolution causing 25 % of the protein to be lost into solution over 1 hour. Air drying for 1 hour had the same effect as freeze drying demonstrating that the cross-linking process was not complete after 1 hour. Freshly made cables showed a 50 % loss in mass after 1 hour, although a

washing step could be much shorter than this. The experiment demonstrates that freshly made cables cannot be stored under water and that severe dehydration could potentially be used as a method of stabilisation for cables which are to be inserted into a wound site.

There appears to be a wide spread of data, represented by the error bars, for freshly prepared samples, 1 hour air dried and freeze dried samples. Each block of material was made from fine micron-diameter fibrils which were bound together tightly when dry but were only loosely bound when the material was wet. The variation in this fibril binding when the material was wet led to different rates of dissolution. All the fibrils were tightly bound in the severely dehydrated materials making dissolution difficult. Assay of the initial starting solution showed that there was no detectable Factor XIII present. Factor XIII is a transglutaminase which stabilises proteins by inter-fibrillar crosslinking. Its absence from the solution meant that dehydration must be the cause of the stabilisation seen.

The time for washing freshly prepared samples should be kept to a minimum, < 10 minutes, to reduce solubilisation. Dehydration should be considered as a method for prolonging the material's life *in vitro* and *in vivo* experiments, providing that there is no deleterious effect of dehydration on the cable's ultrastructure and cell adhesion properties. Promising results have been seen where fibronectin materials were stabilised with copper ions without cytotoxicity towards Schwann cells or dermal fibroblasts (Ahmed *et al.*, 1998 a, e). Consideration must be given to the nature of the repair that is required and whether the material must be in place for weeks or months to serve its cell attachment / orientation purpose.

4.5.4. Moisture absorption

Most fibres are hygroscopic, that is they are able to absorb water vapour from a moist atmosphere and conversely desorb or lose water in a dry atmosphere (Booth, 1983). Since the fibronectin material is brittle when dry and extrudes when wet it is useful to know the water absorption capabilities of the material for reference when processing.

Regain and moisture content are both measures of the amount of water in a material sample. Regain is defined as the weight of water in a material, expressed as a percentage

of the oven dry weight, whereas moisture content is the weight of water in a material expressed as a percentage of the total weight. The hygroscopic properties of the material are demonstrated in Figure 4.24 where the material's water absorption - desorption properties are displayed by a hysteresis curve. Water was tightly bound to the material and difficult to remove causing the desorption curve be higher than the absorption curve for any given relative humidity. At relative humidities greater than 65 %, the material started to absorb moisture and the mass increased. This was the point from which the material may be drawn down to the required diameter. For material already containing a significant amount of moisture the relative humidity must be less than 40 % before drying can occur. This is important for freshly made material. Peanut fibres, wool and cotton have all demonstrated a hysteresis relationship with respect to water absorption (Traill, 1945).

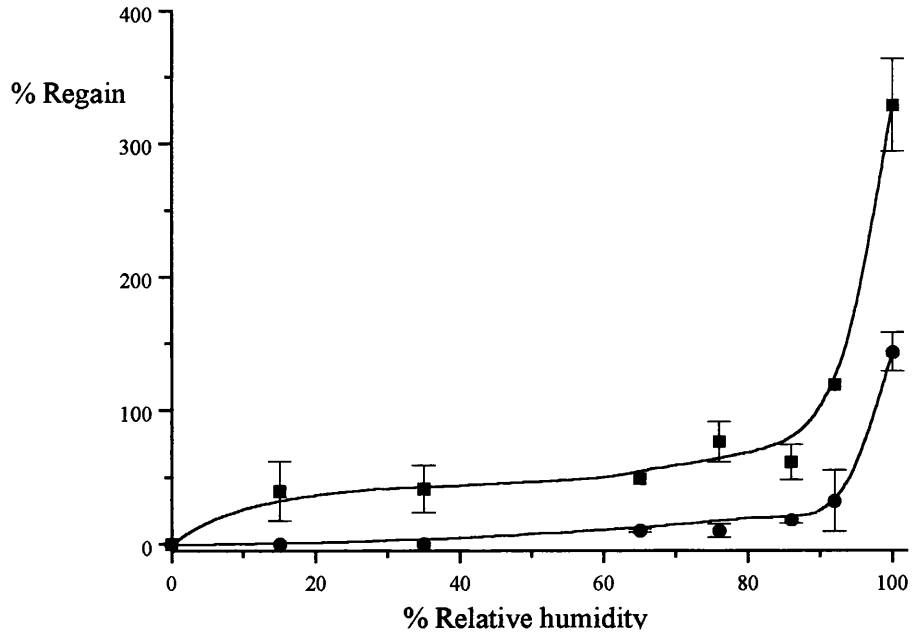


Figure 4.24. *Moisture absorption - desorption curve for protein material with increasing humidity.* ● *- uptake of water by freeze dried material;* ■ *- loss of moisture from freshly prepared material. Regain is the wet weight of water in the material expressed as a percentage of the material's oven dry weight. Results shown are an average of three experiments. Error bars represent 95 % confidence intervals.*

The effects of ionic strength of cell culture medium and incubation temperature on fluid absorption are shown in Table 4.3. Following initial manufacture the material was found to have a moisture content of 70-80 % (regain 325 %) and could thus be drawn immediately to the required diameter. The material was then dried and rehydrated at either room temperature (18°C) to represent a washing step or at 37°C to represent implantation into a wound site. Water and Dulbecco's minimal Eagles medium (DMEM) were used to rehydrate the material, again to compare a washing step to a physiological situation. Rehydration with DMEM was greater than with water at both 18 and 37°C. Thus, for a washing step, during the processing of the material, water would be expected to be taken up into a dry material. A physiological strength solution will also be taken up in *in vitro* culture experiments and *in vivo* usage the cables. When the water molecules enter the cable they penetrate between the micron-diameter fibrils and cause transverse swelling. Axial swelling should be minimal (Booth, 1983). Thus assuming that the cable was cylindrical in shape and that the length of the cable would change only minimally during rehydration, the increased water content of the cable would be expressed as an increase in diameter. Repeated rehydration in the rehydration - dehydration cycle did not affect the results obtained.

Treatment	Moisture content (%)	Rehydrated weight (% freshly drawn weight)
DMEM 37°C	80 ± 1	75 ± 6
Water 37°C	72 ± 5	53 ± 3
DMEM 18° C	76 ± 1	89 ± 8
Water 18°C	78 ± 3	54 ± 8

Table 4.3. *Moisture content of material and rehydration in either water or Dulbecco's minimal eagle medium (DMEM) at 18°C or 37°C. Results are expressed as an average of 3 repeats of 3 dehydration-rehydration cycles with ± 95 % confidence intervals (C.I). The average moisture content for all material was 77 % ± 2 but the actual value for each set of samples is stated in the first column. Rehydrated mass is expressed as a % of the initial mass of freshly drawn material.*

4.5.5. Birefringence

Birefringence was used to test the internal alignment of the material. The angle of rotation of the polarizer required to alter the specimen illumination from maximum to complete extinction was $60^{o} \pm 3$ %. Both twisted and non-twisted specimens gave the same result indicating that internal alignment was also present after twisting.

4.6. Summary

The aim of this work was:

- to identify an alternative method of production of fibronectin-rich templates which could be used as substrates for orientated cell attachment
- to identify a manufacturing method which was reproducible, had low material wastage, and had potential for scale-up.

Fibronectin cables with approximate diameter 200 μm could be drawn from solutions of mixed composition, typically 65 % fibronectin and 35 % fibrinogen when the pH was lowered to less than 5.0. Only the 5-10 μm diameter strands, previously described by Ejim *et al.* (1993), could be drawn from the affinity chromatography purified fibronectin solutions. Thus purified solutions of fibronectin are not essential, or even desirable, for the production of fibronectin materials for tissue engineering. Removal of the affinity chromatography step from a manufacturing process reduces the cost and simplifies the process.

Mechanism of production: A reduction in pH is believed to open out the fibronectin molecule to form an extended configuration. This allows greater inter-domain and inter-molecule interaction.

A reduction in pH to less than 5.0 led to the formation of fibrous precipitates in fibronectin / fibrinogen solutions. This precipitate was formed by isoelectric precipitation, the neutralisation of molecular surface charges leading to molecular aggregation. The pH of protein minimum solubility was found to be around pH 3.0,

lower than published values of pI for either protein.

Optimisation of cable production: Citric acid precipitated out the protein without the extensive aggregation seen with hydrochloric acid. Cables could be drawn from a solution containing 4.8 mg/mL total protein, composition 65 % fibronectin / 35 % fibrinogen, when the total protein concentration was diluted down to 3.2 mg/mL with 0.1 M citric acid. The final pH was 4.0-4.5. At first, 60 % of the total protein was precipitated but drawing cables up from the solution caused more protein to precipitate out and when no further cables could be drawn the final protein concentration in solution was 20 %. This however is wasted protein.

Cable properties: Protein cables consisting of both fibronectin and fibrinogen and being made of numerous micron-diameter fibrils were drawn up from precipitated protein solutions. These cables could be drawn down to smaller diameters when wet but were strong and brittle when dried. The cables were hygroscopic and swelled transversely when placed in solutions of either water or growth medium. The stability of these materials to dissolution could be improved by extensive dehydration.

These cables have been shown to support the attachment and alignment of human dermal fibroblasts, *in vitro*, for distances up to 900 μm away from the cable (Harding, 1996). Since these cables have poor workability, they could be supported on backing materials such as collagen, hyaluronan, alginate and used to orientate sheets of cells (see chapter 7). This would reduce the amount of fibronectin required and remove the problem posed by the brittle characteristics of the material.

Drawing cables is not a technique for large scale manufacture. These results have been used to develop an alternative manufacturing technique based on traditional textile manufacturing methods which will produce materials with the same cell adhesive and orientating properties but with potential for large scale processing.

5.0. Development of a wet spinning technique for fibronectin fibres

5.1. Introduction

The drawn fibres described in chapter 4.0 were made by a process deemed non-scaleable. They were found to be brittle but had cell adhesion properties suitable for use as a tissue engineering scaffold (Harding, 1996). This chapter aims to identify a potential wet spinning process for the production of fibronectin / fibrinogen fibres. The scale of this process can then be increased and the properties of spun fibres examined (chapter 6.0).

The aim of wet spinning is to extrude the protein solution or dope through an orifice, called the spinneret, into a coagulation bath, which precipitates the protein into a fibre. Traditionally, fibres spun from protein have consisted of purified proteins, extracted from their source by acid precipitation. This precipitate was then dissolved in sodium hydroxide and extruded into an acidic solution to form continuous fibres. The exact strength of the solvent and composition of the acid bath depends upon the protein being spun (see section 1.8.3) (Moncrieff, 1969). However after fibre formation, in either the food or textile industry, the protein fibres are usually judged on texture or physical properties rather than their cell binding / protein binding functions.

This chapter investigates the concentration of protein required for wet spinning fibronectin/ fibrinogen fibres, the dope viscosity and a number of different coagulation conditions. The conditions chosen must lead to the formation of stable fibres whilst maintaining the cell adhesion properties of the cables.

5.2. Concentration / viscosity for fibre production

Fibres cannot be spun from low concentration, pure protein solutions. The solution must contain enough protein for cohesive forces to promote protein molecule interaction and fibre formation. Young and Lawrie (1975b) spun porcine lung and stomach proteins from a solution with protein concentration 97.5 mg/mL and bovine plasma proteins from

solutions with concentration 85-98 mg/mL. Concentrations of > 300 mg/mL, (Balmaceda and Rha, 1974) 100-250 mg/mL (Huang and Rha, 1971) and 200-300 mg/mL (Traill, 1945) have been quoted for spinning zein, single cell protein and ground nut protein respectively.

The protein concentration of the solution used to draw cables was 5 mg/mL with a viscosity of 0.01 Poise at 23°C. A protein concentration step was required to increase the solids content and viscosity of the solution before it could be used for wet spinning protein fibres.

5.2.1. Concentration

Protein solutions were made up in phosphate buffer for the cable manufacture process. The maximum total protein concentration which could be achieved by dissolving protein / PEG precipitate into the buffer was 18-20 mg/mL. Further concentration of protein by ultrafiltration was discounted due to the large protein losses on the concentrating membrane seen when preparing solutions for fibronectin mat making. Instead an effective protein solvent was used to directly dissolve the fibronectin / fibrinogen PEG precipitate.

To increase the protein concentration to form a spinnable solution, urea was used as a solvent. Lundgren (1949) states that coagulation of proteins during spinning has been recognised as involving both denaturation and aggregation. Homandberg (1987) has reported that extremes of pH or urea can result in the fibronectin molecule's configuration altering from the compact to the extended form and this change in conformation would allow the component chains to aggregate into a fibrous form.

The maximum concentration of fibronectin and fibrinogen which would dissolve in up to 3 M urea was measured. Increasing the concentration of urea in solution led to an increased solubility of protein precipitate Figure 5.1. With urea concentrations of 1 M and 3 M, protein concentrations of 3.3 % and 7.6 % (w/v) (33 and 76 mg/mL) were achieved.

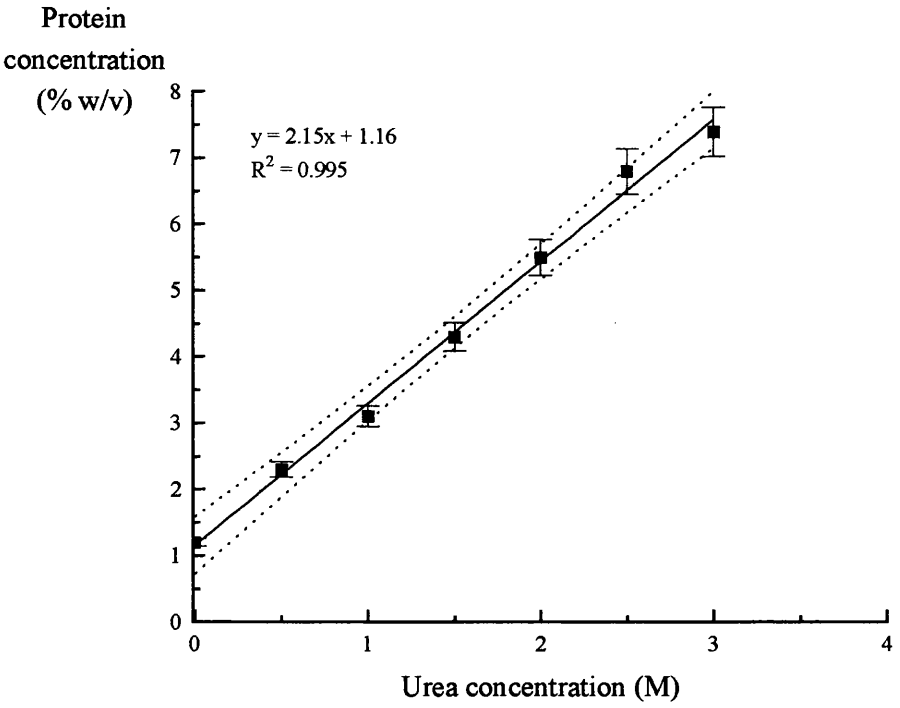


Figure 5.1. *Dissolution of fibronectin / fibrinogen PEG precipitate in urea, concentration 0-3 M. For the 0 M urea sample precipitate was dissolved in 0.02 M phosphate buffer. Error bars represent the error in the protein assay used to measure dissolved protein and dashed lines represent the 95 % confidence band for the data.*

The expected protein concentration after dissolution of PEG precipitate in 6 M urea was 14 % (w/v) (140 mg/mL), calculated by linear regression. Markovic *et al.* (1983) have reported that the regions of fibronectin devoid of disulphide bonds, such as the cell binding domains, may be irreversibly affected by urea treatment, whereas other regions of the molecule are much more stable. Since the material will be finally used as a cell adhesion substrate it is imperative that the structure of the cell adhesion sequences are preserved. Therefore it is important that the concentration of urea used as a solvent is not increased beyond that required for good fibre formation. A urea concentration of 6 M was chosen because it is below the concentration of urea occasionally used to elute fibronectin from affinity chromatography columns (Morgenthaler *et al.*, 1984).

Urea has also been used previously as a solvent for groundnut protein solutions (Traill, 1945) although his work eventually focused on sodium hydroxide as a solvent. Senti *et al.* (1945) state that urea can be used as a solvent for protein spinning if the protein chains are not broken down into lengths with molecular weight less than approximately

15 kDa. Mosesson *et al.* (1975) attributed the decrease in fibronectin's sedimentation velocity seen in the presence of 7 M urea to partial denaturation rather than dissociation of subunits with this effect on the motility of fibronectin molecules being reversed by the removal of urea by dialysis. In contrast, the sedimentation velocity of fibrinogen did not change in the presence of urea. From these results it appears that aqueous urea solutions, probably up to a concentration of 8 M, can be used as a solvent for protein.

Another potential protein solvent is guanidine hydrochloride (GdnHCl). Previously GdnHCl has been used as a denaturing agent, in preference to urea, due to the chemical modification of amino acid chains by cyanate ions contained in the urea solutions. More recently high purity urea has become available and eliminated this problem (West *et al.*, 1997). Alexander *et al.* (1979) report measurement of fluorescent parameters of fibronectin in 6 M guanidine hydrochloride as those of a disordered polypeptide. Major protein unfolding was believed to begin with concentrations of 1.6 M GdnHCl. West *et al.* (1997) compared the effect of urea and GdnHCl on lysozyme and carbonic anhydrase activity and conformation. The enzymic activity of lysozyme was reduced to 50 % of maximum with 0.12 M GdnHCl or 5.6 M urea. Carbonic anhydrase was more sensitive and only 0.3 M GdnHCl or 2.5 M urea were required to halve the enzymic activity. Urea is an uncharged molecule and had no effect on the conformation of lysozyme between 0-10 M concentrations whereas GdnHCl is charged and its denaturing actions are enhanced by it's salt-like properties causing a conformational change in lysozyme between concentrations 3.5-6 M. Due to its potency as a protein denaturant, a concentration of GdnHCl lower than that needed of urea would be required to dissolve high concentrations of protein precipitate.

Sodium hydroxide has been widely used as a solvent for the large scale production of fibres. Although it is known that fibronectin forms an elongated structure under alkaline conditions (Benecky *et al.*, 1991) the effect of high pH on the functional properties of the protein, including cell adhesion, are unknown.

Fibronectin solubility increases at body temperature, 37°C. However the decrease in viscosity seen at a higher temperature (section 5.2.2.) prevents this being used as a method for increasing the solids content of the spinning dope.

5.2.2. Viscosity of concentrated solutions

One aim of increasing protein concentration was to increase the solution viscosity. Figure 5.2. shows the concentration versus viscosity profile for 65 % fibronectin / 35 % fibrinogen solutions. As the protein concentration increases the viscosity increases. At low protein concentrations (0 - 30 mg/mL, 0 - 3 % w/v) this relationship is linear but a concentration is reached, termed the characteristic concentration, when the protein molecules start to interact and the viscosity increases exponentially with increasing concentration. For the fibronectin / fibrinogen solution this characteristic concentration is reached at approximately 3 % (w/v). For bovine serum albumin, lysozyme and β-lactoglobulin concentrations of 17 %, 12 % and 10 % were recorded (Menjivar and Rha, 1980). These high values were achieved with pure protein solutions. Bell (1982) found a value of 5 % for a soya protein solution and discussed the possibilities of impurities in the solution reducing this critical concentration.

The viscosity of the fibronectin / fibrinogen solution was also temperature dependent, Figure 5.2. Increasing the temperature from 23°C to 37°C led to a viscosity reduction from 17.6 mPa s (0.17P) to 13.1 mPa s for a 60 mg/mL solution and a decrease from 67.3 to 41.8 mPa s for a 98 mg/mL solution. Decreasing the temperature to 4°C led to an increase in viscosity. For 5 mg/mL and 60 mg/mL solutions the viscosity increased from 1.0 mPa s to 1.7 mPa s and 17.6 to 62.4 mPa s respectively. With high protein concentrations, > 80 mg/mL, decreasing the temperature to 5° C resulted in the formation of gels that were difficult to spin and whose viscosity was difficult to measure accurately (a 98 mg/mL solution had viscosity of 221 mPa s at 4°C). Homandberg (1987) reported that prolonged incubation of fibronectin at low temperature or ionic strength resulted in fibril formation and Brown *et al.*(1987) used monoclonal antibodies to detect small conformational changes in the fibronectin molecule at 4°C and 37°C. They concluded that a conformational change on cooling might involve the self-aggregation of fibronectin, seen at low temperatures. Such self-aggregation would increase the solution viscosity. Increasing the concentration of the protein would increase the likelihood of such interactions and exacerbate this effect, as seen in Figure 5.2 (lower).

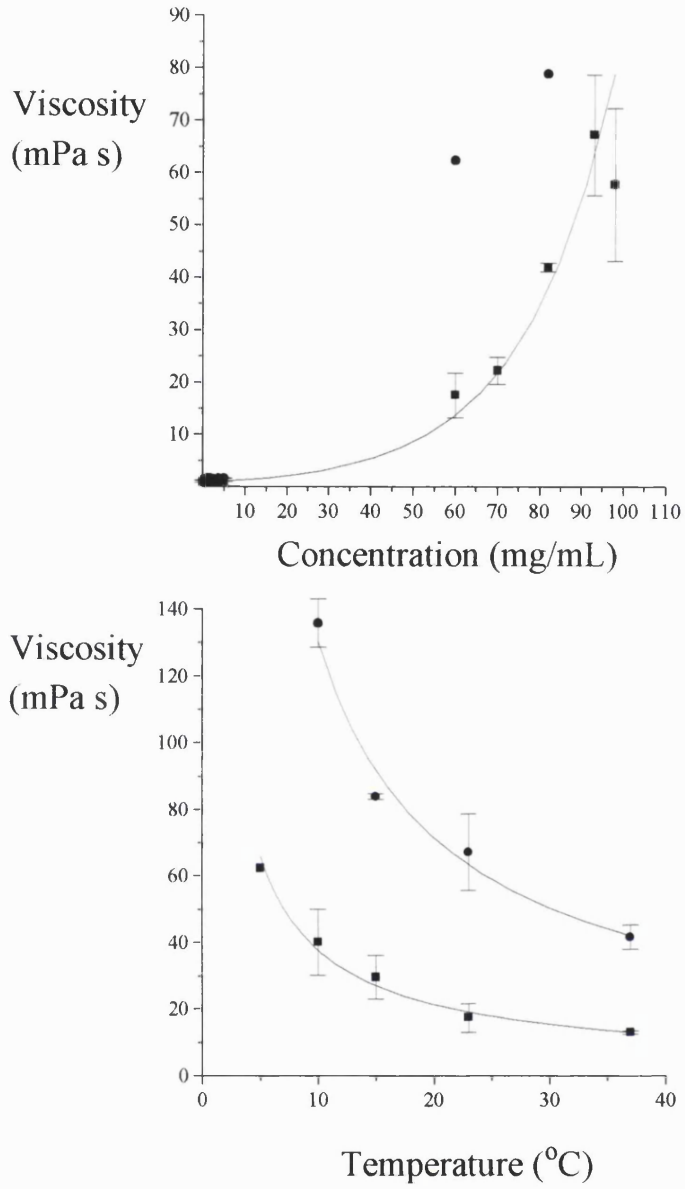


Figure 5.2. *Protein concentration versus apparent viscosity (above). Viscosity was measured at 23°C, ■, for solutions of total protein concentration 0-98 mg/mL, (65 % fibronectin, 35 % fibrinogen) and at 4°C, ●, for solutions with total protein concentration 60 and 82 mg/mL. Solutions with protein content 0 - 5 mg/mL were dissolved in phosphate buffer, whilst solutions with a protein concentration greater than 60 mg/mL were dissolved in urea.*

Solution apparent viscosity versus temperature (below). Measurements were made at 5, 10,15,23 and 37°C for solutions with total protein concentration 60 mg/mL, ■, and 93 mg/mL,●. For both graphs, results are the average of at least two samples and error bars represent the 95 % confidence interval for the data. Measurements for solutions with concentration 1-5 mg/mL (shear rates 150-877s^{-1}),70 and 82 mg/mL (24-877s^{-1}) were made using the concentric cylinders. The DIN 125 cup and bob was used for 60 mg/mL, 93 and 98 mg/mL solutions (shear rates 39.9-492s^{-1}).

There will also be some increased interaction between the fibronectin and fibrinogen in solution at 4°C. Stathakis *et al.* (1978) reported fibronectin binding to fibrinogen columns at 4°C but little binding at 22°C. No cryoprecipitation occurs when only fibronectin and fibrinogen are present but at high concentrations of these two proteins may increase interactions and increase viscosity. No measurements were made to investigate the presence of yield stress at low shear rates. Interest was concentrated on higher values of shear rate which may mimic those seen on extrusion from the spinneret.

Fibronectin / fibrinogen solutions were found to be Newtonian with the up curve equal to the down curve for the range of shear rates tested. For 5 mg/mL solutions, n = 1.02 ± 0.06 (95 % confidence interval) and 0.98 ± 0.07 at 23 and 4°C respectively. For 60 mg/mL solutions, n = 1.14 ± 0.03 and n = 1.08 ± 0.10 at 23 and 4°C respectively. Both solvents, phosphate buffer and 3 M urea were also Newtonian. Errors in measurement of viscosity and power law parameters are discussed in Appendix 2, section 10.2.3.

5.2.3. Addition of viscosity enhancers

A recommended range of solution viscosities for wet spinning is 25 - 380 Poise (P) (Young and Lawrie, 1974). Fibres have been spun from a bovine plasma protein solution with a viscosity of 250 P and porcine lung and stomach proteins with apparent viscosities 2 - 7 P (shear rate 517s^{-1}) (Young and Lawrie, 1974, 1975a). Producing a solution with a suitable viscosity is important for homogeneous mixing and extrusion of the protein as well as deaerating the solution before extrusion.

5.2.3.1. Choice of viscosity enhancers

Solutions of fibronectin / fibrinogen with protein concentration 98 mg/mL had a viscosity of 60-70 mPa s (0.6-0.7 P) at 23°C. To increase the viscosity to a level suitable for spinning, the possibility of adding a viscosity enhancer was investigated. A number of possibilities were chosen and are listed in Table 5.1. The pH of all the additives was tested to ensure that they did not precipitate out the protein on mixing.

Polyethylene oxide (PEO) is a high molecular weight polyethylene glycol derivative. It is non-toxic and will give a high viscosity solution at a low concentration, Table 5.2.

Sorbitol is a carbohydrate which is frequently used in plasma processing to protect proteins from denaturation during pasteurisation. Dextran is a non-toxic, high viscosity starch and glycerol is a well-characterised high viscosity, non-toxic liquid.

Sodium Carboxymethylcellulose (CMC) and sodium alginate are both high viscosity, anionic polysaccharides. CMC has been tested for its potential use in separating haem from red blood cells (Yang and Lin, 1996) and has been tested as a topically applied gel on lower leg ulcers in diabetic patients (d'Hemecourt *et al.*,1997). Alginate is frequently used as a wound dressing (Agren, 1996), has been used to impregnate developmental vascular grafts (Lee *et al.*,1997) and has been described in a chemically modified form as a potential tissue engineering scaffold (Bouhadir *et al.*, 1998). Alginate sponges for use in cell transplantation have also been described (Shapiro and Cohen, 1997).

The rheological properties of all the chosen additives were characterised for the range of concentrations stated in Table 5.1 and the results given in Figures 5.3 - 5.6 and Table 5.2.

Additive	Concentration range tested (% w/v)	pH range
Polyethylene oxide (PEO) ($M.W.6x10^4$)	0.5 - 1.0	6.0 - 6.3
Sorbitol	10 - 40	6.3
Sodium Carboxymethyl cellulose (CMC)	4 - 8	6.6 - 6.9
Sodium alginate	1 - 5	6.3 - 6.6
Dextran ($M.W. 2x10^6$)	20 - 40	6.9
Glycerol	60	5.0

Table 5.1. *Range of viscosity enhancers tested and the pH of the solutions when prepared with deionized water. pH was measured using a range of indicator papers, (Whatman, Maidstone, UK)*

The aim was to find an additive that could be added at a low concentration and would raise the viscosity of the fibronectin solution to a suitable spinning viscosity without compromising the final use of the material as a tissue engineering scaffold.

Sodium alginate is a shear thinning solution. The power law exponent, n, for 1 % sodium alginate is 0.9 but the shear thinning behaviour increases with increasing alginate concentration (Figure 5.3). For a 5 % (w/v) solution, n = 0.6. Since the solution is non-Newtonian, viscosity is dependent upon shear rate. Apparent viscosity comparisons are made for values at $1000s^{-1}$ to represent the shear inside the spinneret (see 6.2). Solutions of 1 % and 5 % alginate have apparent viscosities of 24 mPa s (0.24 P) and 532 mPa s respectively at room temperature. Alginate dissolved well in 3 M urea. The 3-5 % (w/v) alginate solutions were extruded into a hydrochloric acid / calcium chloride bath and precipitated to form stable fibres. The 1 and 2 % (w/v) solutions also precipitated but did not form stable fibres. The same result was seen on extrusion into sulphuric acid / sodium sulphate baths although the quality of fibre was not as good. When soluble sodium alginate is contacted with a calcium salt, the cations exchange and insoluble calcium alginate is formed. This reaction is reversible. If fibres were spun containing alginate, soaking the fibres after formation in a solution of a sodium salt should remove the polysaccharide, if required. Doyle *et al.* (1996) have shown mixed results when examining the effect of calcium alginate on wound healing processes. Calcium alginate was found to increase proliferation of fibroblasts but decrease proliferation of human microvascular endothelial cells and keratinocytes. It was also found to decrease fibroblast motility but it had no effect on either keratinocyte motility or on the formation of capillary-like structures by the endothelial cells. It has also been shown to activate macrophages in the wound environment, which may play a role in wound healing (Thomas *et al.*, 1997). These effects are believed to have been mediated by the calcium ions.

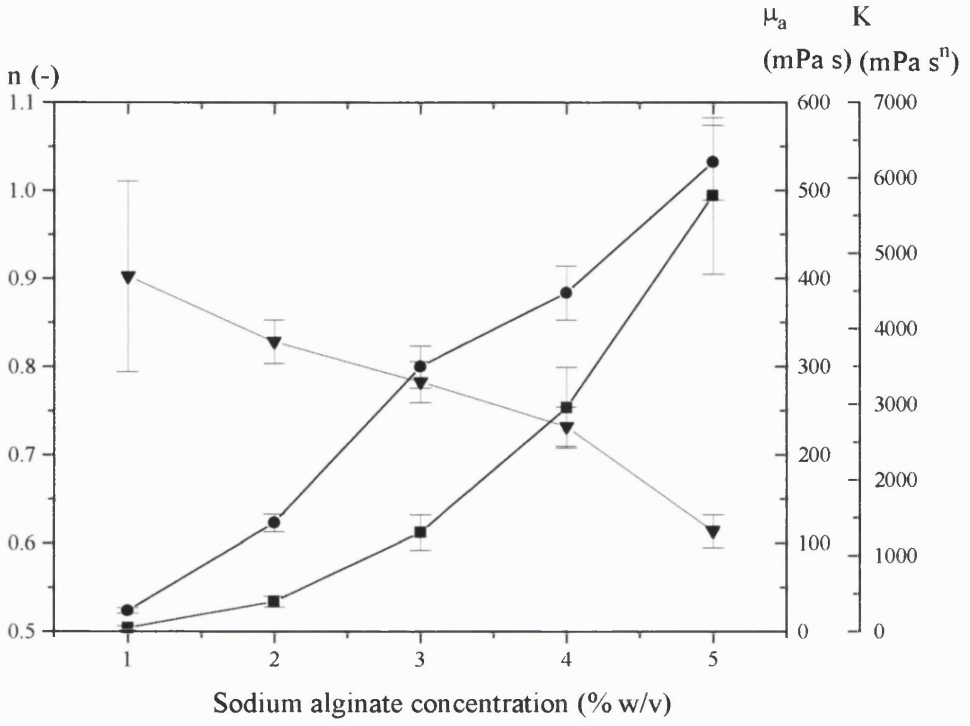


Figure 5.3. *Rheological parameters for sodium alginate solutions, concentration 1-5 % (w/v). Values for power law exponent, n (-)* ▼*; consistency index, K (mPa* s^n*),* ■ *and apparent viscosity,* μ_a*, at a shear rate of 1000*s^{-1}*,* ●*. Rheology of 1 % sodium alginate solution was measured using the concentric cylinders (apparent viscosity given for shear rate 877*s^{-1}*, shear rate range tested, 49.8 - 877*s^{-1}*). For concentrations 2-5 %, measurements were made using the cup and bob 125, shear rate range 6.65 - 1007.2*s^{-1}*. Values shown were obtained from the up curve at 23°C. Errors are the maximum error of the mean for the measuring device used (see 10.2.3).*

Dextran (Figure 5.4.) shows Newtonian behaviour for 20-30 % (w/v) concentrations whilst 40 % dextran exhibits shear thinning behaviour over the range of shear rates tested (20 % w/v tested between 19.5 and 1007s^{-1}, 30-40 % w/v tested between 6.7 and 1007s^{-1}). At 23°C, 20 % dextran had an apparent viscosity of 137 mPa s (1.37 P) at a shear rate of 1000s^{-1} and n = 1.03. Doubling the dextran concentration to 40 % (w/v) resulted in an apparent viscosity of 1242 mPa s (12.4 P) and n=0.95. Dextran did not form a stable precipitate when extruded into acid.

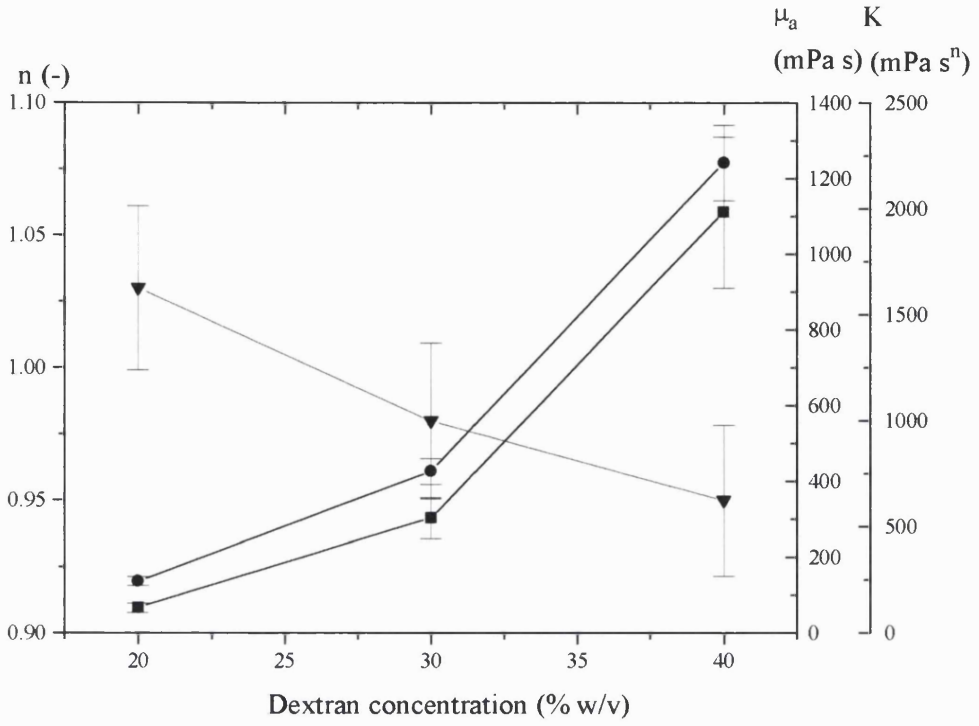


Figure 5.4. *Rheological parameters for dextran solutions, concentration 20-40 % (w/v). Values for power law exponent, n (-)* ▼, *consistency index, K (mPa s^n),* ■, *and apparent viscosity, μ_a, at a shear rate of 1000s^{-1}* ●. *Values were obtained from the up curve at 23°C and measurements were made with the DIN 125 cup and bob. Error bars are the maximum error for this measuring device.*

The rheology of 10-40 % (w/v) sorbitol solutions, Figure 5.5, was measured for the range of shear rates 49.8 - 877s^{-1}. Apparent viscosities were low, 1.1 mPa s for a 10 % (w/v) solution and 2.3 mPa s for a 40 % solution and the solutions were Newtonian.

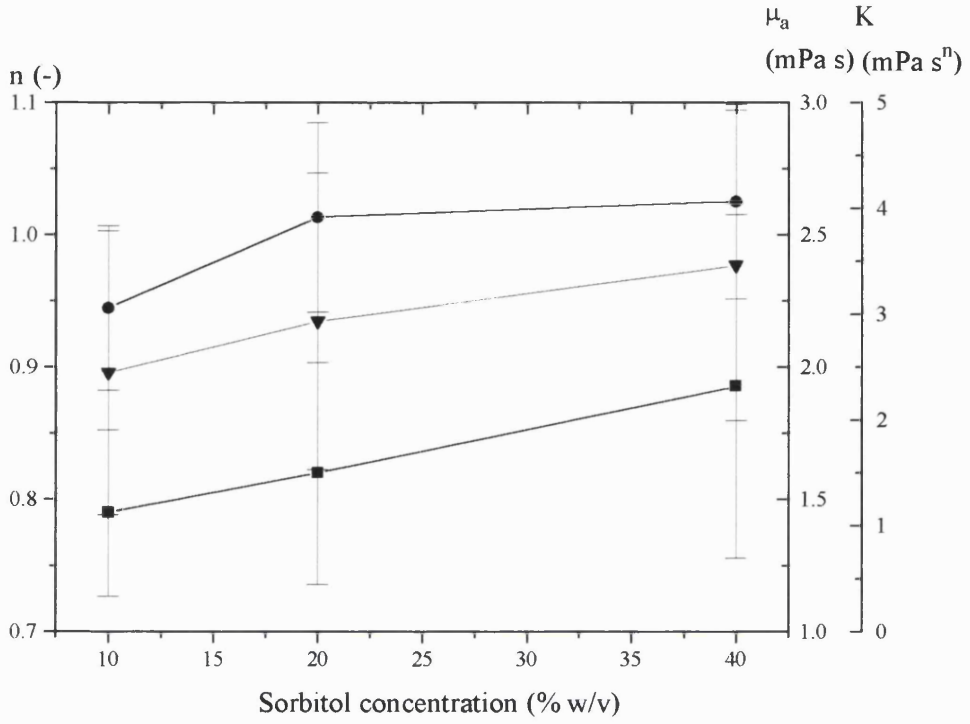


Figure 5.5. *Rheological parameters for sorbitol solutions, concentration 10-40 % (w/v). Values for power law exponent, n (-)* ▼*; consistency index, K (mPa s^n),* ■*, and apparent viscosity, μ_a, at a shear rate of 877s^{-1},* ●*. Values were obtained from the up curve at 23°C and measurements were made in the concentric cylinders for all solution concentrations. Error bars are the maximum error of the mean calculated for the concentric cylinders for each parameter (Appendix 2, 10.2.3).*

Sodium Carboxymethylcellulose (CMC) can be purchased in a range of different viscosity grades. Increasing the CMC concentration was found to increase the shear thinning behaviour of the fluid (Figure 5.6) with n = 1.07 and 0.90 for the 4 and 8 % solutions respectively. It would be expected that the measured value for n for the 8 % solution would be lower than that for the 6 % solution however this difference was not seen and may be due to the use of the 125 measuring system for the 4 and 6% solutions and the 114 measuring system for the 8 % solution. Apparent viscosity increased from 89 mPa s (0.89 P) to 258 mPa s when the CMC concentration was increased from 4 % to 8 %. CMC has been shown to have potential clinical uses and must therefore be purchasable as a clinical grade product. To reduce the amount of CMC which would be required to raise the viscosity, a high viscosity CMC was also tested, Table 5.2. This CMC was highly shear thinning at a concentration of 0.5 % and had an apparent viscosity of 47 mPa s at a shear rate of 704s^{-1}. CMC solutions did not form stable precipitates in acid.

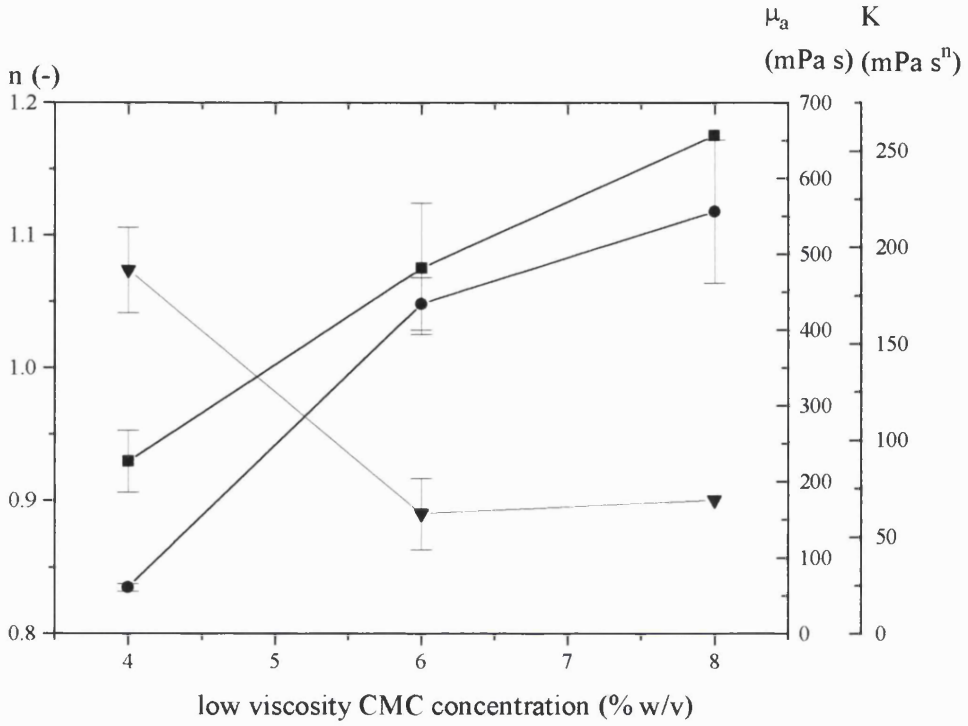


Figure 5.6. *Rheological parameters for CMC solutions, concentration 4-8 % (w/v). Values for power law exponent, n (-) ▼; consistency index, K (mPa s^n), ■, and apparent viscosity, μ_a, at a shear rate of 1000s^{-1} ●. Values were obtained from the up curve at 23°C. For the 4 % solution, the tested shear rate range was 19.5-1007.2s^{-1} and 6.65 - 1007.2s^{-1} for the 6-8 % solutions. The DIN 125 measuring device was used for the 4 and 6 % (w/v) solutions and the DIN 114 device for the 8 % solution. Errors for the equipment were large (372 and 22 %) for the K and n values respectively for the 8 % solution and have been excluded for clarity.*

Low concentrations of a high molecular weight PEG derivative, PEO, were also shear thinning, n = 0.76 and 0.61 for 0.5 % and 1.0 % solutions respectively. A 1 % PEO solution was more viscous than both the 1 % alginate or the 0.5 % high viscosity CMC solution. PEO also did not precipitate out of solution when extruded into acid.

Solution	n (-)	K (mPa s^n)	Apparent viscosity, μ_a, at 1000s^{-1} (mPa s)	Shear rate range (s^{-1})
0.5 % PEO	0.76	92	22	27.9 - 1007.2
1.0 % PEO	0.61	575	51	6.65 - 1007.2
0.5 % high viscosity CMC	0.58	801	47 (at 704s^{-1})	6.65 - 704
60 % glycerol	1.12	5	29	81.8 - 1007.2

Table 5.2. *Rheological parameters for other solutions which were considered as viscosity enhancers for spinning solutions. Measurements were taken from the up curve at 23°C. Shear rate range used depends upon solution viscosity and measuring device.*

From these results, three viscosity enhancers were tested at low concentrations with fibronectin / fibrinogen precipitate. Sorbitol and glycerol were not chosen because of the need to have a high concentration to give a high viscosity. Dextran was not tested because its relatively Newtonian properties combined with its high viscosity made it difficult to dissolve the fibronectin precipitate although at lower concentrations this problem may be eliminated. Low concentrations of the shear thinning and moderate viscosity additives alginate, high viscosity CMC and PEO were chosen. None of these solutions formed stable fibres when extruded into acid and therefore fibres formed would be due to a combination of protein and additive.

It is estimated that when fibres are extruded through the spinneret they will be exposed to high shear. Since the viscosity of a shear thinning solution decreases with increasing shear rate, this high shear in the spinneret will reduce the viscosity of the extruded fluid. This will reduce the pressure drop over the spinneret and thus the pumping power required in the system but will also affect the viscosity on extrusion into the coagulant and the final properties of the fibre.

5.2.3.2. Selected additives and fibronectin

The three chosen additives, 1 % PEO, 1 % sodium alginate and 0.5 % CMC were dissolved in 3 M urea with protein precipitate to give a final protein concentration of

60 mg/mL. The precipitate composition was 65 % fibronectin / 35 % fibrinogen. The viscosity of the solution was measured at 37, 23 and 10°C on the day of manufacture and after 24 hours storage at room temperature. The 24 hour viscosity test was used to examine whether spinning viscosity could be maintained if the solution was left to degas for 24 hours. The absorbence of the solution at 410 nm (A410) was measured spectrophotometrically to determine the solution turbidity before and after the 24 hour period and to examine the effect of passing the solution through a glass fibre filter on clarification. If the protein / additive dope was to be used to spin very fine fibres, e.g.: < 100μm, then the solution would have to be pre-filtered to prevent undissolved material blocking the spinneret holes.

1 % PEO

The 1 % PEO dissolved well in 3 M urea along with the fibronectin / fibrinogen precipitate. The resulting solution was shear thinning, with apparent viscosity decreasing with increasing shear rate. Increasing temperature led to a decrease in solution viscosity and a decrease in shear thinning behaviour. However solutions did appear to have relatively stable viscosity after 24 hours storage at 23°C, Figure 5.7. This figure shows curves fitted to the data using equation 5.3. Values for n, K and the regression coefficient, R^2 are also shown.

Power law equation:

$$\tau = K\gamma^n \qquad \text{(equation 5.1)}$$

Since:

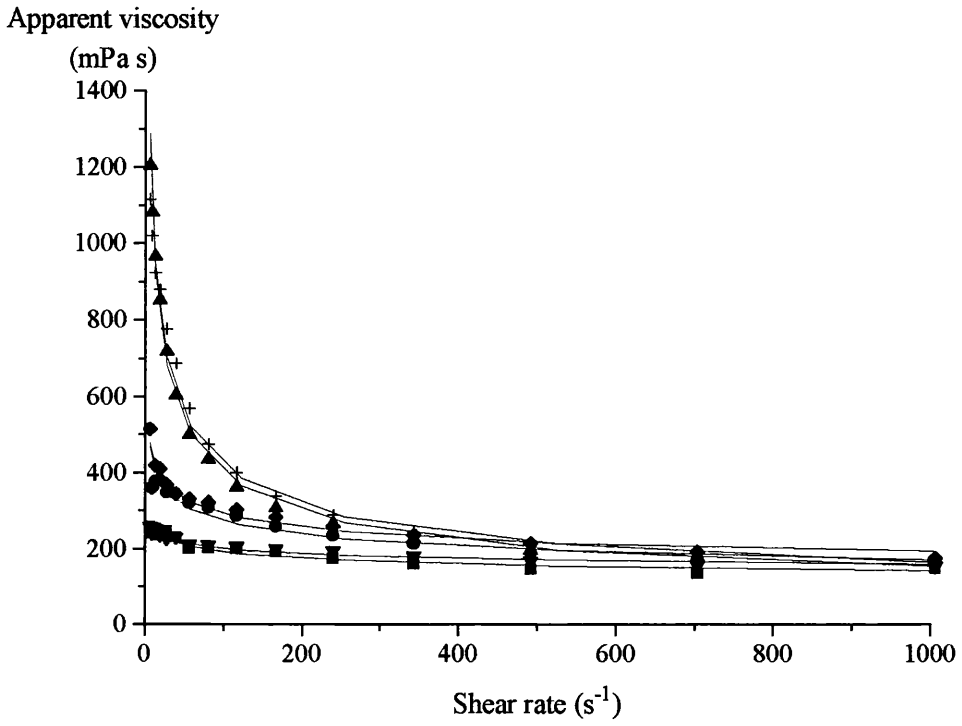
$$\frac{\tau}{\gamma} = \mu_a \qquad \text{(equation 5.2)}$$

then:

$$\mu_a = K\gamma^{n-1} \qquad \text{(equation 5.3)}$$

Measurements of solution turbidity were made for 1 % PEO and 60 mg/mL protein on days 1 and 2. On day 1, A410 was 2.6 and on day 2, 4.2. Values for a 60 mg/mL protein only solution were 1.2 and 2.7 respectively. The values in the presence of PEO are greater than for protein alone suggesting that there is undissolved protein present in solution, possibly as a result of PEO precipitation.

Extrusion of solutions into a hydrochloric acid / $CaCl_2$ bath at room temperature resulted in the formation of stable fibres both before and after overnight storage and showed that fibres could be spun from a solution at room temperature. Decreasing the temperature to 10°C, although increasing the viscosity, may lead to gel formation at low shear rates, for example, in the pressure reservoir.

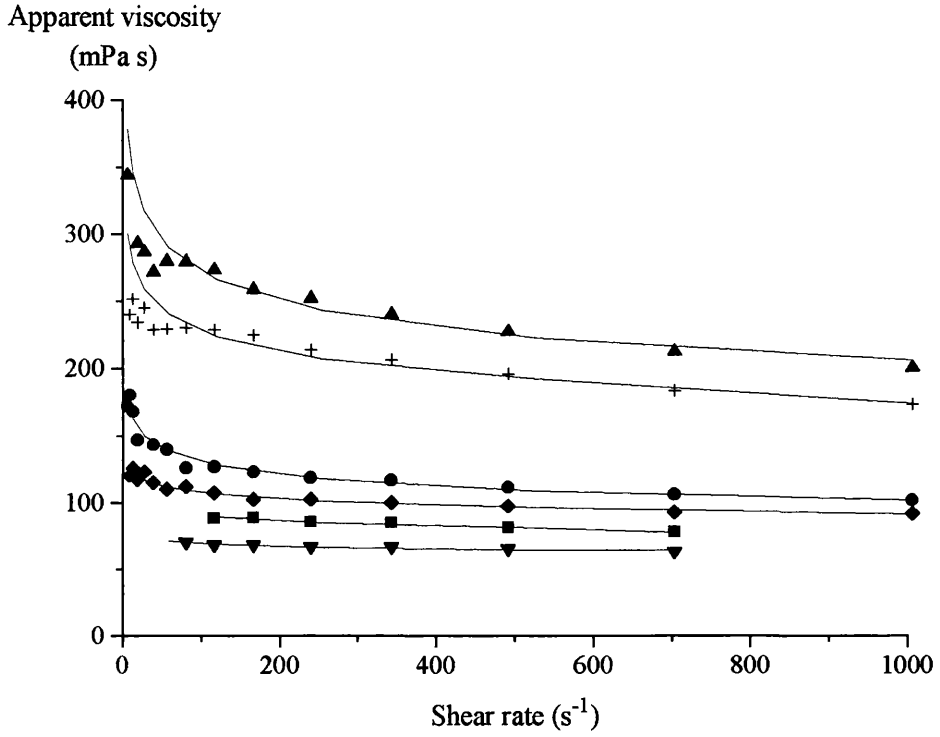


Day / Temp	K (mPa s^n)	n (-)	R^2
Day1 37°C	348	0.87	0.929
Day1 23°C	680	0.80	0.945
Day1 10°C	2813	0.58	0.995
Day2 37°C	318	0.90	0.953
Day2 23°C	677	0.82	0.938
Day2 10°C	2790	0.59	0.989

Figure 5.7. *Solution apparent viscosity versus shear rate for 1 % PEO + 60 mg/mL protein. Viscosity at 37, 23 and 10°C after manufacture or after storage at 23°C for 24 hours.* ■ *- 1 % PEO + 60 mg/mL protein, day 1, 37°C;* ● *- day 1, 23°C;* ▲ *- day 1, 10°C;* ▼ *- day 2, 37°C;* ◆ *- day 2, 23°C; + - day 2, 10°C. Viscosity was measured for the range of shear rates 6.65-1007s^{-1} (data for up curve only shown) and curves were fitted according to equation 5.3, with the regression coefficient, R^2, indicating how well the curve fits the data. Since results are comparative no error bars have been included.*

1 % sodium alginate

Figure 5.8. shows the effect of shear rate, temperature and storage time on the apparent viscosity of a 1 % sodium alginate, 60 mg/mL fibronectin / fibrinogen solution.



Day / Temp		K (mPa s^n)	n (-)	R^2
Day1	37°C	123	0.93	0.891
Day1	23°C	204	0.90	0.967
Day1	10°C	474	0.88	0.960
Day2	37°C	84	0.96	0.927
Day2	23°C	148	0.93	0.967
Day2	10°C	399	0.88	0.977

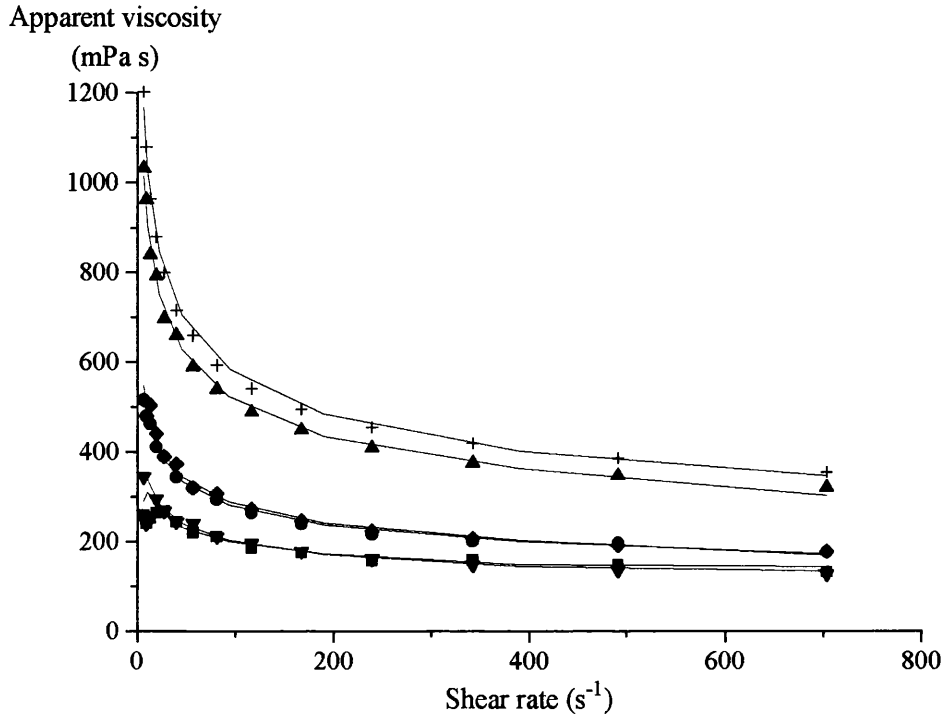
Figure 5.8. *Solution viscosity versus shear rate for a 1 % sodium alginate plus 60 mg/mL protein. Solution viscosity at 37, 23 or 10°C was measured on the day of manufacture (day1) and after 24 hours storage at 23°C.* ■ *- 1 % alginate + 60 mg/mL protein, day 1, 37°C;* ● *- day 1, 23°C;* ▲ *- day 1, 10°C;* ▼ *- day 2, 37°C;* ◆ *- day 2, 23°C; + - day 2, 10°C. Data from the up curve only was fitted over a range of shear rates 57.2 - 1007s^{-1} (117 - 1007s^{-1} for 37°C samples) using equation 5.3. Regression coefficients,R^2, are given to indicate the extent of curve fit.*

The protein / alginate mixture, like the protein / PEO mixture, was also shear thinning, with an increase in shear thinning behaviour with a decrease in temperature to 10°C. However, on day 1 measurements taken at 10°C gave n = 0.88 for the alginate / protein solution and 0.58 for the PEO / protein solution. Thus mixtures containing 1 % PEO have greater shear thinning properties than those containing 1 % sodium alginate. For 1 % PEO, at shear rates $1000s^{-1}$, the apparent viscosity for solutions at all temperatures is very similar, due to the increased shear thinning behaviour with decreased temperature. The 1 % alginate solution shows a higher apparent viscosity (at $1000s^{-1}$) at 10°C and 23°C, than at 37°C but lower apparent viscosities than the 1 % PEO solutions. There was a decrease in viscosity of the 1 % alginate / 60 mg/mL protein solution after overnight storage but stable, non-self adherent fibres could be spun from solutions on both days.

Solution turbidity, A410, was measured for the protein and alginate solutions before and after filtration through a glass fibre syringe mounted filter. Values for before and after filtration on day 1 were 2.4 and 0.8 respectively. The value for the pre-filtered solution on day 2 increased from 2.4 to 3.8. This may again be due to protein precipitation during storage or bacterial infection (see section 3.6). Fibres containing sodium alginate could be spun both pre and post filtration and there was no measured loss in protein concentration during filtration, suggesting the removal of only non-dissolved particulate material.

<u>0.5 % CMC</u>

0.5 % CMC solutions were also shear thinning and showed the same temperature-dependent relationships as 1 % PEO and 1 % sodium alginate, Figure 5.9. The power law exponent, n, for the 10°C mixture on day 1 was 0.74. In a similar manner to 1 % sodium alginate / protein solutions and unlike 1 % PEO solutions, the apparent viscosity of the solutions at $1000s^{-1}$ is temperature dependent. There was no loss in rheological stability with 24 hours storage at room temperature and the sample measured at 10°C showed a viscosity increase on day 2. Solutions could be passed through a filter, reducing the turbidity measurement on day 1 from 2.4 to 2.0. Again the turbidity measurement of the pre-filtered solution increased on day 2, to 3.8. Stable fibres could be spun from these solutions into a hydrochloric acid / calcium chloride bath.



Day / Temp	K (mPa s^n)	n (-)	R^2
Day1 37°C	386	0.85	0.892
Day1 23°C	831	0.76	0.995
Day1 10°C	1660	0.74	0.998
Day2 37°C	464	0.81	0.904
Day2 23°C	877	0.75	0.989
Day2 10°C	1911	0.74	0.998

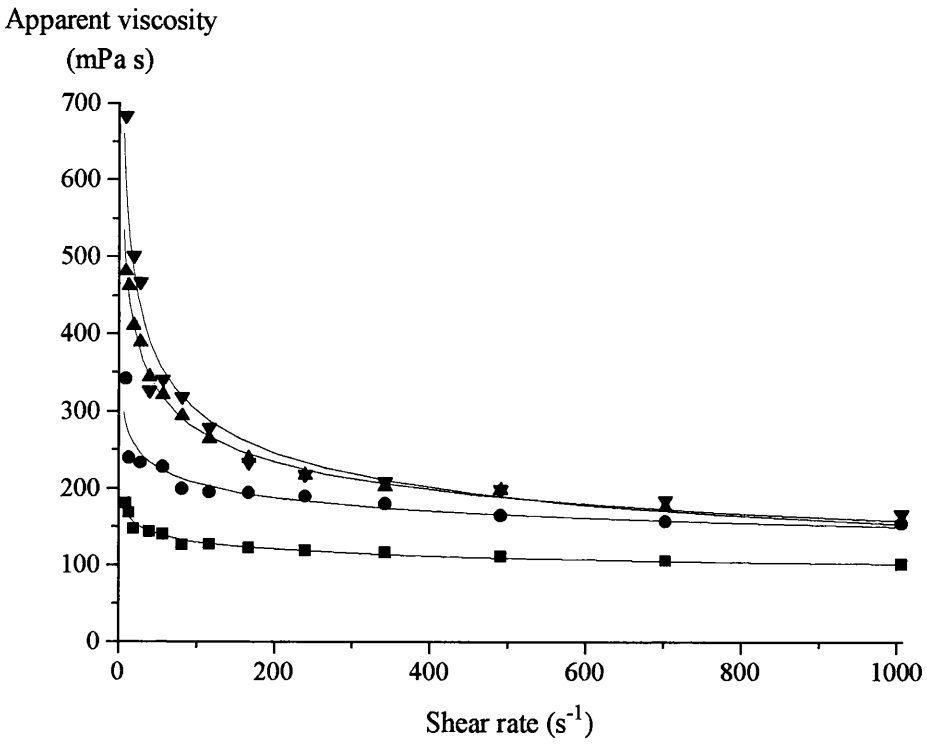
Figure 5.9. *Solution apparent viscosity versus shear rate for 0.5 % CMC + 60 mg/mL protein. Viscosity at 37, 23, or 10°C after manufacture or after storage at 23°C for 24 hours.* ■ *- 0.5 % CMC plus 60 mg/mL protein, day 1, 37°C;* ● *- day 1, 23°C;* ▲ *- day 1, 10°C;* ▼ *- day 2, 37°C;* ◆ *- day 2, 23°C;+ - day 2, 10°C. Viscosity was measured for the range of shear rates 6.65-1007s^{-1} (data for up curve only) and curves were fitted using equation 5.3. Regression coefficients for the fit are given, R^2.*

No stable fibres could be spun from a fibronectin / fibrinogen solution, concentration 60 mg/mL, without additives. At least 70 mg/mL of protein was required to spin protein only fibres. Thus addition of viscosity enhancers allows fibre spinning at lower protein concentration although the proportion of protein and additive present will affect the cell adhesion and physical properties of the fibre.

The effect of increasing protein concentration further by dissolving the protein / PEG precipitate in 6 M urea and adding either 1 % sodium alginate or 0.5 % CMC is examined in Figure 5.10.

The total protein concentration was increased to 88 mg/mL by dissolving the solutions in 6 M urea. With increasing protein concentration, the consistency index, K, increased, representing increased viscosity. The apparent viscosity of the two 88 mg/mL solutions with either alginate or CMC was similar at a shear rate of $1000s^{-1}$, approximately 175 mPa s (1.75 P) (Figure 5.10). Thus on extrusion, at a high shear rate, the solutions will have the same rheological properties but on storage or mixing, at a low shear rate, the CMC solution would tend to be more viscous. These solutions are of a lower viscosity than those recommended as suitable spinning solutions but both form stable fibres when extruded into an acid bath. Both solutions can also be passed through a glass fibre pre-filter.

Despite the decrease in stability of the alginate solutions overnight, further work was carried out with this additive due to the formation of fibres with no self-adherent properties and the previous history of the use of alginate as wound dressings. Alginates are typically used as wound coverings rather than supports for cell attachment. By using only 1 % alginate it was hoped that the cell adhesion properties of the protein would not be compromised. The addition of CMC will also be studied due to its moderate viscosity and potential medical applications, described in section 5.2.3.1.



Protein conc.(mg/mL) /additive	K (mPa s^n)	n (-)	R^2
60 / 1% alg	204	0.90	0.967
88 / 1% alg	359	0.88	0.937
60 / 0.5% CMC	845	0.76	0.958
88 / 0.5% CMC	1144	0.71	0.958

Figure 5.10. *Shear rate versus apparent viscosity for solutions with protein concentration either 60 or 88 mg/mL with 1 % alginate or 0.5 % CMC as a viscosity enhancer.* ■ *- 3 M urea, 60 mg/mL protein, 1 % alginate;* ● *- 6 M urea, 88 mg/mL protein, 1 % alginate;* ▲ *- 3 M urea, 60 mg/mL protein, 0.5 % CMC;* ▼ *- 6 M urea, 88 mg/mL protein, 0.5 % CMC. All measurements were made at 23°C and are taken from the up curve. Curves were fitted for a shear rate range 39.9-1007s^{-1} for solutions containing alginate and 9.5-1007s^{-1} for those containing CMC. Regression coefficients, R^2, are given for the curve fit.*

Addition of additives is a potential method for increasing the viscosity of the spinning solution. Factors such as precipitation of the additive in the coagulation bath, interaction of protein and additive, the effect of shear thinning behaviour on extrusion and acceptability of the additive in the final product have to be considered. Although a great

variety of these additives could be used, in practice, only CMC and in particular alginate will be considered further to make this study more manageable. The following section will apply the success of these findings to produce protein dopes that can be used to wet spin fibres and to optimise the coagulation conditions.

5.3. Coagulation bath composition

The function of the coagulation bath is to precipitate the protein from the dope and remove the solvent. Ineffective removal of the solvent can lead to poor fibre properties with respect to stability and strength. Coagulation baths rarely consist of only one component, containing most frequently both an acid and a salt. This section aims to investigate a number of precipitants for their inclusion as part of the coagulation bath.

5.3.1. Solubility curves with various salts

Sections 4.2. and 4.3. described acid and salt precipitation of fibronectin / fibrinogen solutions with respect to cable drawing. Figures 5.11 - 5.16 show solubility curves for precipitants such as PEG, ethanol, ammonium sulphate and a number of other salts.

Polyethylene glycol is the non-ionic water soluble polymer used in the preparation of the fibronectin / fibrinogen precipitate (see section 3.3) and is widely used in the plasma fractionation industry. Figure 5.11 shows the solubility curve for PEG 4000 showing both the decrease in soluble protein with increasing concentrations of PEG and the resulting increase in solution pH. This increase in pH was unexpected and contrasts observations made during fibronectin enrichment of cryoprecipitate with PEG, where the supernatant of PEG precipitated solutions usually displayed a slight decrease in pH. Accuracy in pH measurement was believed to be $\pm$ 0.1 pH units and therefore this pH change is perhaps not significant. In all cases the increase in volume caused by the addition of extra salt was accounted for. These solubility experiments were performed with much lower total protein concentrations than would be used for wet spinning, 5 mg/mL compared to at least 70 mg/mL, due to material availability. The error bars shown are for the error of the protein assay and are greater than the error found between duplicated, experimental samples.

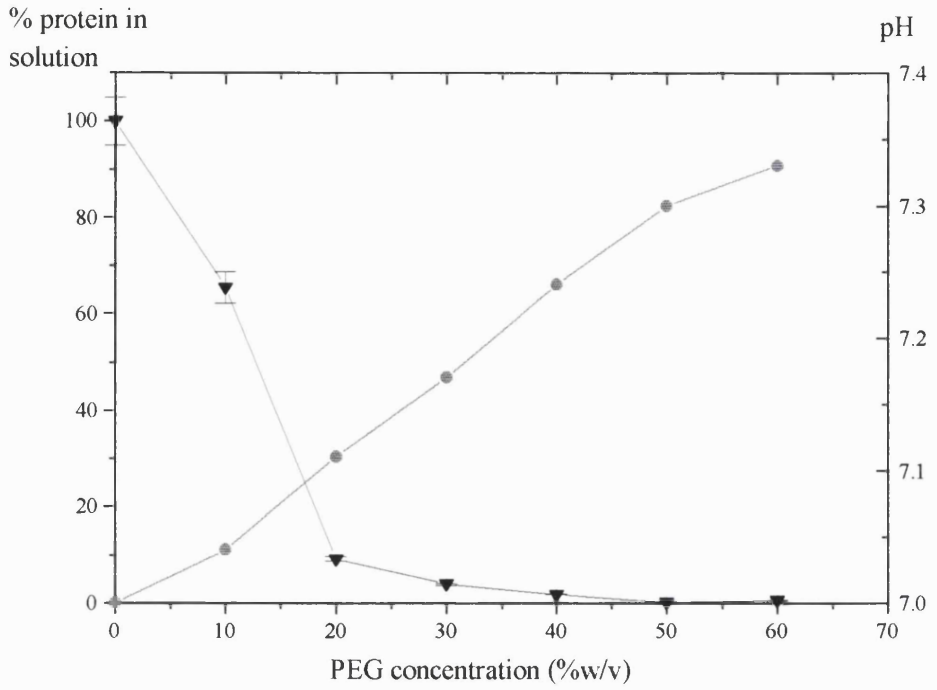


Figure 5.11. *Solubility of protein on the addition of polyethylene glycol 4000 (PEG). Measured amounts of PEG were added to 4 mL solution containing 3.4 mg/mL fibronectin and 1.3 mg/mL fibrinogen. Reduction of protein in solution,* ▼, *was measured after centrifugation using the Bio-Rad protein assay. Error bars represent the assay error. Solution pH was also measured,* ●. *All measurements were made at 23°C.*

Increasing the PEG concentration above 20 % (w/v) results in the precipitation of at least 90 % of the protein present in solution. Scopes (1994) states that many plasma components are precipitated before 20 % (w/v) is reached and that solutions of this concentration are of a low enough viscosity to be handleable. However PEG added to a mixed vessel for precipitation purposes can be of higher viscosity than a coagulation bath. Extrusion into a high viscosity bath could result in a high viscous drag on the fibre as it is towed through the bath, increasing fibre orientation, but making precipitation at the spinneret orifice difficult. To test the use of PEG as a coagulation bath, a 70 mg/mL protein solution was extruded into PEG solutions, concentration 4, 10, 25 and 50 % with pH 4.1, 4.1, 4.3 and 4.8 respectively. Precipitation occurred in the 4 and 10 % PEG solutions but stable continuous fibres were not formed. For PEG concentrations 25 and 50 % (w/v), continuous fibres were formed which could be drawn away from the coagulating bath. The density difference between 50 % PEG and the extruded protein solution caused the formed fibres to rise to the surface. The pH of the mixture of protein and PEG is much higher (7.0 - 7.5) than that for PEG alone due to the effect of the

phosphate buffer in which the protein is dissolved. The addition of salts or the alteration of the pH of the PEG solution to approximately the protein's point of minimum solubility may allow lower concentrations of PEG to be used in a coagulation bath.

Ethanol is used in the plasma fractionation industry as an organic protein precipitant. It acts by lowering the solvating power of water. Ethanol has a low dielectric constant, 26 compared to 78 for water (Kistler and Friedl, 1980). Ethanol is highly miscible with water and lowers the dielectric constant of water and also its effective concentration (Englard and Seifter, 1990). As a result proteins are precipitated mainly by dipolar and electrostatic interactions (Scopes, 1994). Figure 5.12 shows the solubility curve for ethanol at 4°C. Ethanol's high heat of solution requires that solutions are chilled whilst precipitation occurs to prevent protein denaturation. Concentrations of ethanol > 30 % (v/v) are required to precipitate out the fibronectin / fibrinogen mixture. The pH rose from 7.0 - 7.5 with an increase in ethanol concentration. In a similar manner to PEG addition, the use of ethanol precipitation would be improved by the manipulation of both the pH and an increase in the ionic strength of the coagulation bath.

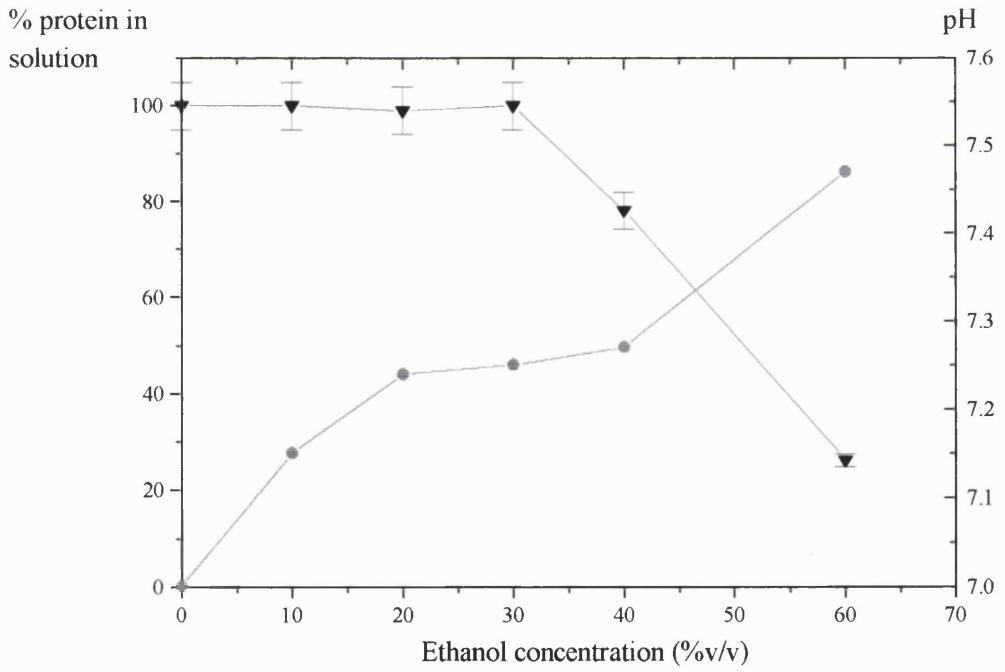


Figure 5.12. *Protein solubility with the addition of ethanol at 4°C. Measured volumes of ethanol were added to fibronectin / fibrinogen solutions to give final ethanol volumes 0-60 % (v/v). Reduction of protein in solution,* ▼*, was measured, after centrifugation, using the Bio-Rad protein assay. Error bars represent the assay error. Solution pH was also measured,* ●.

Salt based coagulation baths

In solution proteins are folded so that most of their charged residues are on the surface and their uncharged residues are folded away from the solvent. Addition of high concentrations of salt neutralises these surfaces charges and allows the protein molecules to aggregate (Englard and Seifter, 1990). The extent to which this happens depends upon the nature of the salt. The Hofmeister series lists the salting out ability of anions. Multiple-charged anions such as citrate, phosphate and sulphate have greater salting out ability than monovalent anions such as chloride and acetate (Scopes, 1994). Shih *et al.* (1992) write that the ability of an anion to dehydrate a protein is related to the square of its valency, thus polyvalent anions are most effective. Monovalent cations such as ammonium, potassium and sodium are effective precipitants whilst multivalent metal ions tend to form complexes with the protein (Scopes, 1994).

Ammonium sulphate is a commonly used protein precipitant, favoured for its ability to precipitate a wide variety of proteins without denaturing them (Englard and Seifter, 1990). Figure 5.13. is the solubility curve for a 4.7 mg/mL total protein solution at room temperature. Addition of 1 M ammonium sulphate (25 % saturated solution) reduces protein solubility to 0.94 mg/mL. Increasing the salt concentration to 3 M reduces the protein in solution further to 0.25 mg/mL. The pH of 3 M ammonium sulphate is 5.6 although after the addition of 3 M ammonium sulphate to the protein solution the pH of the protein / salt mixture is pH 6.5-6.6., above the pH required for protein precipitation according to the pH solubility curves (section 4.3). Thus the mechanism of precipitation here is only 'salting out'. 3 M ammonium sulphate will be compared quantitatively to other potential coagulation baths in section 5.3.3.

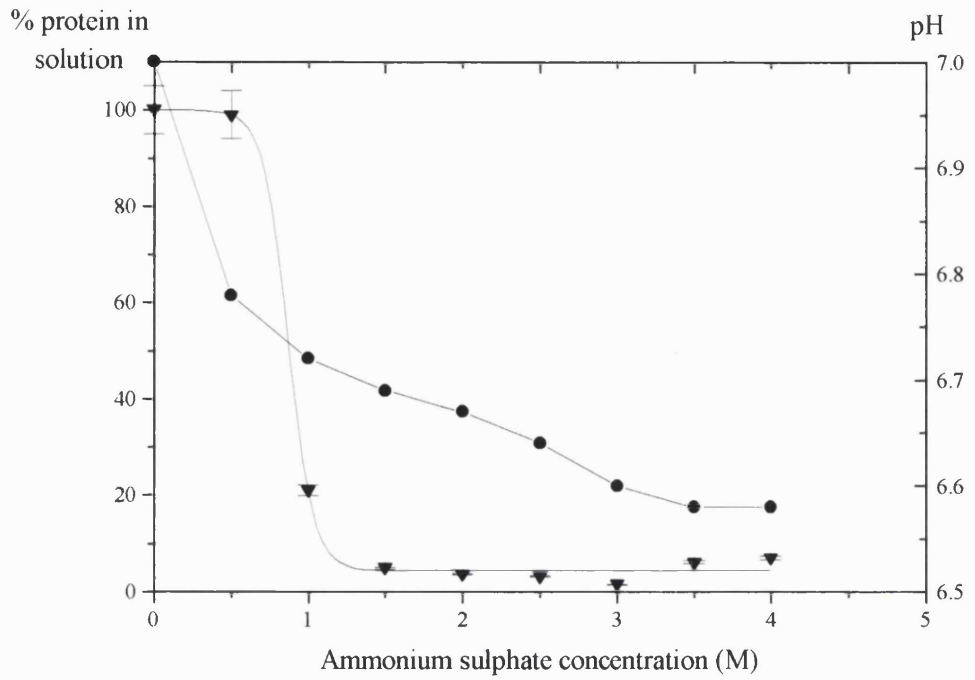


Figure 5.13. *Precipitation of protein from solution with ammonium sulphate. Solid ammonium sulphate was added to 65 % fibronectin / 35 % fibrinogen solutions to give final salt concentrations 0-4 M. The protein remaining in solution,* ▼*, was measured using the Bio-Rad protein assay. Error bars represent the error of the assay. pH was also measured,* ●.

Figures 5.14. and 5.15. show solubility curves for sodium sulphate, tri-sodium citrate, calcium chloride and sodium chloride. According to the Hofmeister series both citrate and sulphate are more effective at precipitating proteins than chloride. Sodium sulphate is not highly soluble at low temperatures (Scopes, 1994) but the salt could be dissolved in the protein solution up to a concentration of 1.1 M (pH 6.9) at room temperature. However, no protein was precipitated until a concentration of 0.3 M was reached. The initial soluble protein concentration, 4.7 mg/mL, was reduced to 1.9 mg/mL with 0.55 M sodium sulphate (60 % reduction in solubility) and 0.5 mg/mL with 1.1 M salt (90 % reduction in solubility). A 0.55 M solution of tri-sodium citrate showed a 90 % reduction in solubility, with an increase in pH to 7.6, demonstrating the superior salting out properties of the citrate ions compared to sulphate ions.

Addition of 1.1 M calcium chloride resulted in a decrease in pH to 4.0. whereas 1.1 M sodium chloride reduced the pH to only 6.7. This reduction in pH with $CaCl_2$ was due to the divalent cation in $CaCl_2$ and the hydrogen ions which are present in solution after its

hydrolysis. NaCl has a monovalent cation and forms both strong acid and base when dissolved. The stoichiometry of these reactions is shown in equations 5.4 and 5.5.

$$CaCl_2 + 2H_2O \Leftrightarrow Ca^{2+} + 2Cl^- + 2OH^- + 2H^+ \qquad \text{equation 5.4}$$

$$NaCl + H_2O \Leftrightarrow Na^+ + Cl^- + OH^- + H^+ \qquad \text{equation 5.5}$$

The precipitating action of sodium chloride does not appear as strong as calcium chloride system. Addition of 1.1 M calcium chloride resulted in 45 % of the protein being precipitated. However only 15 % of protein was precipitated when 1.1 M sodium chloride was added, compared to a 90 % reduction with the addition of 1.1 M sodium sulphate. These results support the Hofmeister sequence that citrate and sulphate ions are more effective at precipitating proteins than chloride ions. It appears that the pH reduction associated with dissolved calcium chloride also aids precipitation. Dilute acidic solutions of salts such as sodium chloride have been used previously to extract fibrous proteins including: fibrinogen, non cross-linked keratin, myosin and collagen (Englard and Seifter, 1990).

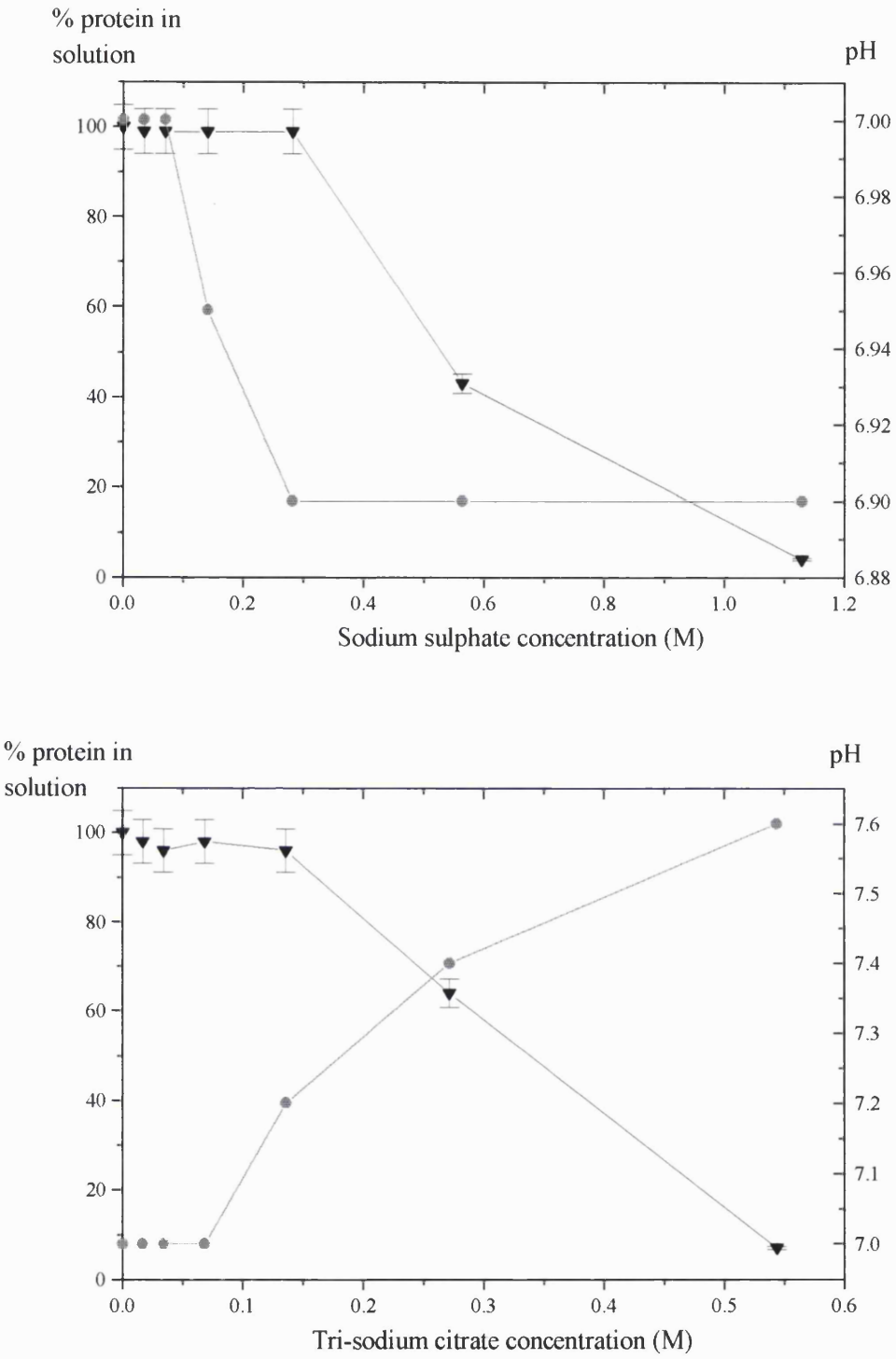


Figure 5.14. *Precipitation of protein from solution with sodium sulphate (above) and tri-sodium citrate (below). Solid salt was added to fibronectin / fibrinogen solutions. The protein remaining in solution, ▼, was measured using the Bio-Rad protein assay. Error bars represent the error of the assay. Solution pH was also measured, ●.*

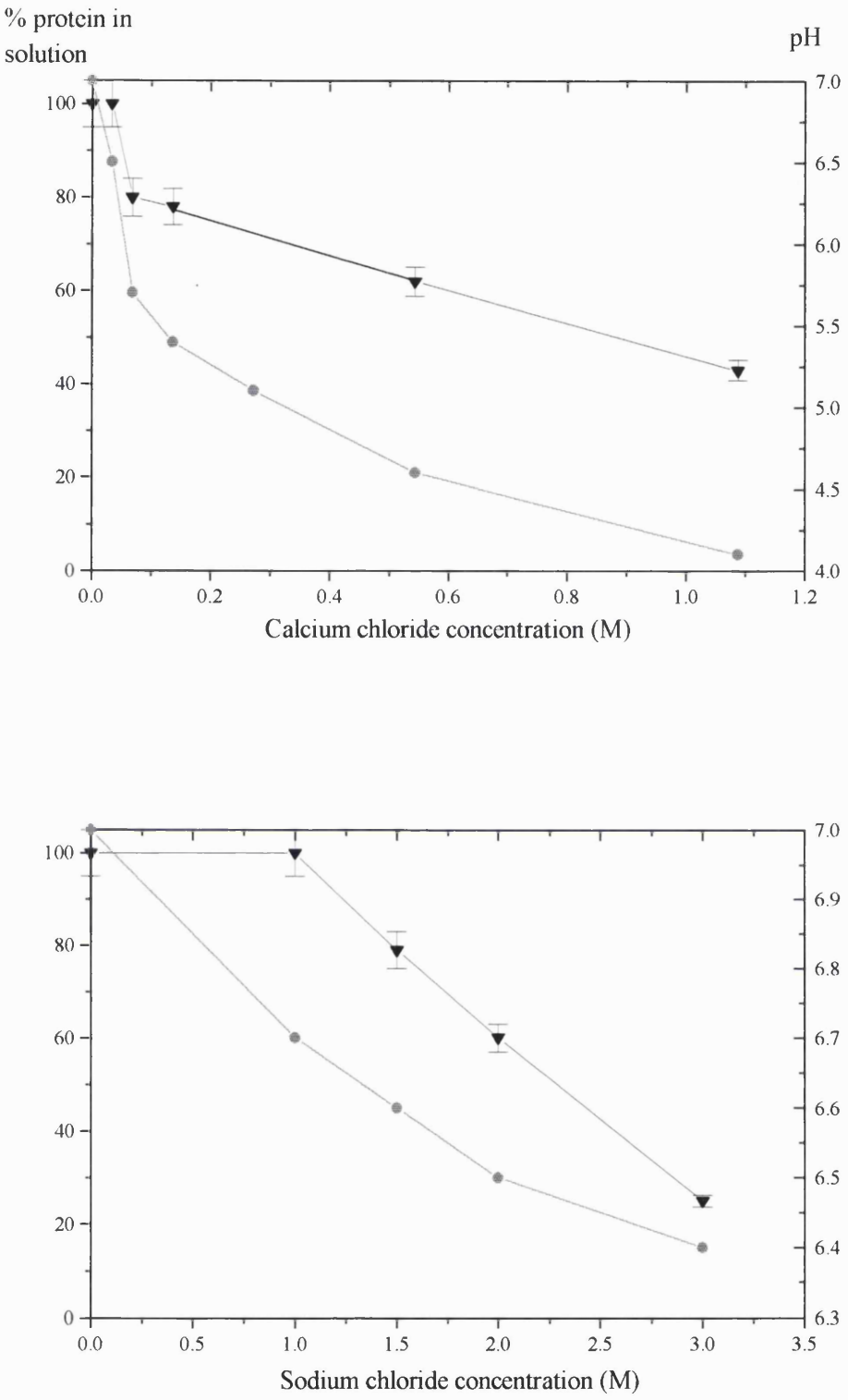


Figure 5.15. *Precipitation of protein from solution with calcium chloride (above) and sodium chloride (below). Solid salt was added to 3.4 mg/mL fibronectin / 1.3 mg/mL fibrinogen solutions. The protein remaining in solution, ▼, was measured using the Bio-Rad protein assay after centrifugation. Error bars represent the error of the assay. Solution pH was also measured, ●.*

Sodium chloride was used in a further experiment to examine the effect of both changing the initial solution pH and the ionic concentration. The results are shown in Figure 5.16.

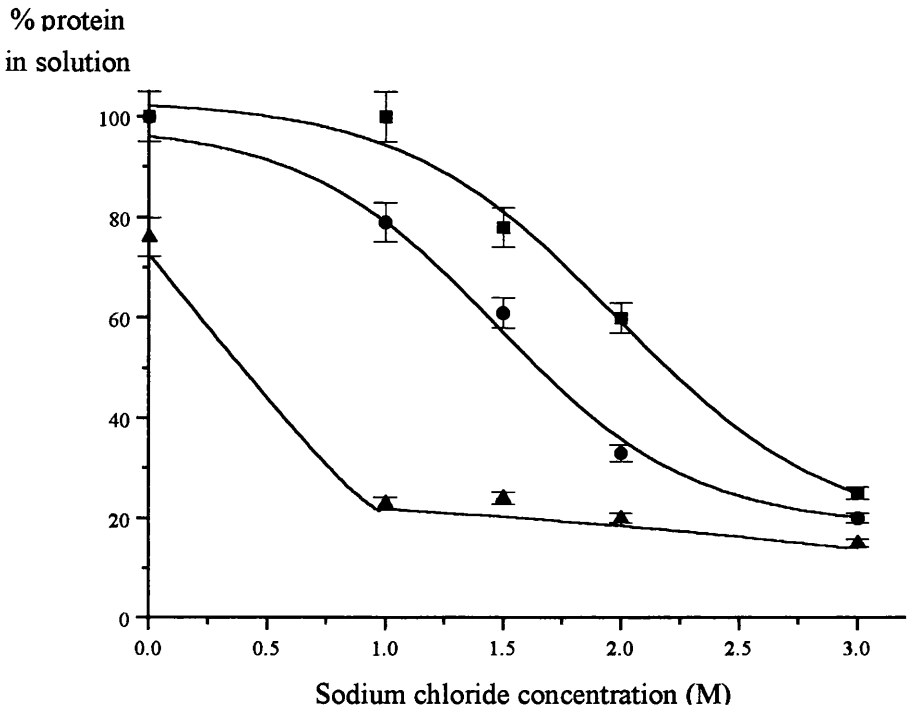


Figure 5.16. *Protein precipitation from solution (initial total protein concentration 4.7 mg/mL) when both solution pH and solution ionic strength are altered. The ionic strength of fibronectin / fibrinogen solutions with an initial pH of 7.0,* ■*; pH 6.0,* ●*; or pH 5.1,*▲*, was increased using solid sodium chloride. The protein remaining in solution was measured using the Bio-Rad protein assay after centrifugation. Error bars represent the error of the assay. The increase in volume on addition of the salt was accounted for.*

The pH of a 3.4 mg/mL fibronectin / 1.3 mg/mL fibrinogen solutions was either left at 7.0, or reduced with HCl to 6.0 or 5.1. Solid sodium chloride was added to each sample and the amount of protein precipitated measured. The pH of the solutions with initial pH 7.0 and 6.0 was reduced to 6.4 and 5.4 respectively on addition of 3 M NaCl. The solution with a starting pH of 5.1 had a final pH of 4.7 after addition of the salt. At pH 7.0 and 6.0 there was no loss of protein in moderately low salt environments (0-1 M). However if the sodium chloride concentration was increased there was both a reduction in pH and an increase in ionic strength, resulting in protein precipitation. If the pH was initially reduced to 5.1 by addition of acid, then 24 % of the protein was precipitated. This figure was confirmed by the solubility curve, Figure 4.5. Increasing the salt concentration in solutions with original pH 7.0, 6.0 and 5.1 from < 0.1 M to 3 M

resulted in a decrease of protein in solution from 100 % to 24, 21 and 14 % respectively. The solubility curves for pH 7.0 and 6.0 samples were fitted with sigmoidal curves whilst that for the pH 5.1 sample could not be fitted to a sigmoidal curve. This difference could be attributed to precipitation for the pH 5.1 sample occurring due to both salting-out and isoelectric precipitation. For the pH 7.0 sample only salting out would have occurred, whilst there may have been a small amount of precipitation due to pH reduction in the pH 6.0 sample, leading to the curve shift to the left. In general, a reduction in pH as well as salt addition results in more protein being precipitated for a solution of a given ionic strength. Development of coagulation baths will look towards combining the salts tested here with acids to produce stable fibres.

5.3.2. Testing a variety of coagulation baths

Solubility curves for solutions containing varying proportions of fibronectin and fibrinogen were shown in section 4.3. There were two regions where protein was precipitated out, at both very low pH, less than 1.0, and the pH of minimum solubility for fibronectin / fibrinogen and their mixtures, between 3.0 and 5.0. At a very low pH this precipitation occurs due at least partially to denaturation, whilst between pH 3.0 and 5.0 the proteins have no overall charge and tend to aggregate.

The aim of wet spinning is to precipitate the protein on its extrusion into a coagulation bath. Figure 5.16. demonstrated the effect of combining both pH and salt concentration to precipitate proteins. Strong acids such as HCl or H_2SO_4 were used to denature the protein whilst weaker acids such as citric acid, acetic acid and glycine were used to create environments at a higher pH. Salts were also used to enhance the precipitation effect. The coagulant should be a non-solvent for the protein and must remove the solvent in which the protein is extruded. In this case the aim was to precipitate the protein and extract the urea from the spinning dope. To test different coagulation baths, a 70 mg/mL solution of protein was extruded into 20 mL of each coagulation bath as shown in Figure 2.2c.

Hydrochloric acid.

0.5 M hydrochloric acid, pH 0.6 gave good fibre formation but coagulation occurred too quickly and the needle tended to block easily. Using 0.1 M HCl, pH 1.15, or a more dilute solution did not result in the production of stable fibres. Addition of a salt such as 15 % (w/v) sodium sulphate to 0.1 M HCl, pH 1.9, improved the precipitation but the density difference between the solution and fibre caused the fibre to rise to the surface, making continuous coagulation difficult. A similar density difference was also seen with 0.1 M HCl + 15 % tri-sodium citrate, pH 5.9. Here the formed fibres rose to the surface of the coagulation bath and formed a sheet. Extrusion into 0.1 M HCl + 15 % $CaCl_2$ formed good fibres but a density difference was still observed. Stable fibres could be formed by combining either 0.1 M HCl and at least 2 % $CaCl_2$, pH 1.1, or 0.25 M HCl and at least 1 % $CaCl_2$, pH 1.0. As the concentration of salt was increased, the precipitated protein tended to coagulate into sheets rather than discrete fibres.

Sulphuric acid.

No stable fibres were formed when protein solution was extruded into 0.25 M sulphuric acid with no salt, pH 1.0. Combinations of > 0.01 M sulphuric acid and 2 % (w/v) sodium sulphate, pH 1.9, were found to form stable fibres. Increasing the acid concentration to 0.5 M with 2 % Na_2SO_4 resulted in needle blocking. Addition of either 1 % or 2 % Na_2SO_4 to 0.05 M sulphuric acid produced solutions with pH 1.5 and 1.6 respectively and led to stable fibre formation. Sulphuric acid and tri-sodium citrate combinations did not produce suitable fibres.

Acetic acid

Acetic acid / sodium chloride combinations were used by Swingler and Lawrie (1977) to spin porcine plasma proteins. Addition of 10 % NaCl to 5 to 10 % acetic acid gave solutions with pH 2.0 - 1.7 but the fibronectin/fibrinogen fibres formed showed strong self-adhesive properties and were difficult to remove from the coagulation bath.

Citric acid

0.1M citric acid was used in the manufacture of fibronectin / fibrinogen cables (see section 4.4). Extrusion into a 0.1M citric acid solution caused precipitation but no stable fibres were formed. Addition of sodium sulphate improved precipitation but the formed precipitate rose to the coagulation bath surface. Poor fibres were formed with the

addition of up to 10 % $CaCl_2$ to 0.1 M citric acid, pH 1.2. 0.1 -1.0 M citric acid/ citrate buffers, pH 3.0 were used to create the pH of minimum protein solubility. Although protein was precipitated, it was difficult to remove individual protein fibres from the coagulation bath.

Glycine.

0.1 M glycine / hydrochloric acid, pH 3.0, also precipitated the protein but resulting fibres were poor.

The most successful coagulation baths tested were those involving strong acids. The acid may enhance the opening up of the molecule initiated by the urea, leading to molecule aggregation and fibre formation. By combining acid and salt, the acid concentration required for precipitation was reduced. The quantitative testing of a number of different coagulation baths is described in section 5.3.3.

5.3.3. Comparison of coagulation baths

To quantitatively compare a number of the more successful coagulation baths, dope was extruded into a small scale spinning bath and the amount of precipitate formed measured by a dry weight method. Results are shown in 3 bar graphs in Figure 5.17.

The top bar graph represents an investigation into the composition of different coagulation baths. The independent t-test was used to compare the mean amount of protein precipitated over 3 spinning trials for each test bath. Precipitation into 0.25 M HCl, 2 % $CaCl_2$ was taken as the standard for comparison. Significantly less precipitate was produced on extrusion into water, phosphate-buffered saline and chilled ethanol at neutral pH ($p = 0.000, 0.003, 0.005$ at the 95 % level of significance). Water and PBS are of too low ionic strength and too high pH to precipitate much protein but reducing the pH of the ethanol may have increased protein precipitation. There was no significant difference between the amount of precipitate formed on extrusion into the other baths although only the HCl and H_2SO_4 baths formed stable fibres. A 1 M citric acid / citrate, 10 % $CaCl_2$, pH 0.8 bath also produced suitable fibres but the amount of precipitate was difficult to measure accurately due to the high background salt level. Precipitation into 50 % PEG was also difficult to measure quantitatively and the fibres were not as stable as those made in the acid / salt baths.

The middle and lower bar graphs examine extrusion into 0.25 M hydrochloric acid, 2 % $CaCl_2$, pH 0.9 and 0.05 M sulphuric acid, 2 % Na_2SO_4, pH 1.8 respectively. Dopes were made up either at a low concentration, 90 mg/mL total protein, with or without a viscosity enhancer or a higher protein concentration, 105 mg/mL, without an enhancer. The enhancers tested were 1 % sodium alginate or 0.5 % sodium Carboxymethylcellulose. Comparison of means used the high protein dope at room temperature as a standard. The amount of precipitate formed on extrusion into HCl was not significantly different for the dopes tested at room temperature or when the baths were chilled to 4°C. All dopes formed good, stable fibres. Those containing alginate were noticeably less self-adherent than those formed from other dopes. Extrusion into sulphuric acid produced more varied results with significantly less fibre being formed from the low protein dope, $p= 0.031$ and 0.042 for samples at 23 and 4°C respectively. Extrusion of the alginate dope at 4°C also produced significantly lower results. When the additives sodium alginate and sodium CMC are extruded into the HCl / $CaCl_2$ bath, calcium ions exchange with the sodium ions to form the insoluble complexes, calcium alginate and calcium CMC. This will not happen in the H_2SO_4/Na_2SO_4 bath.

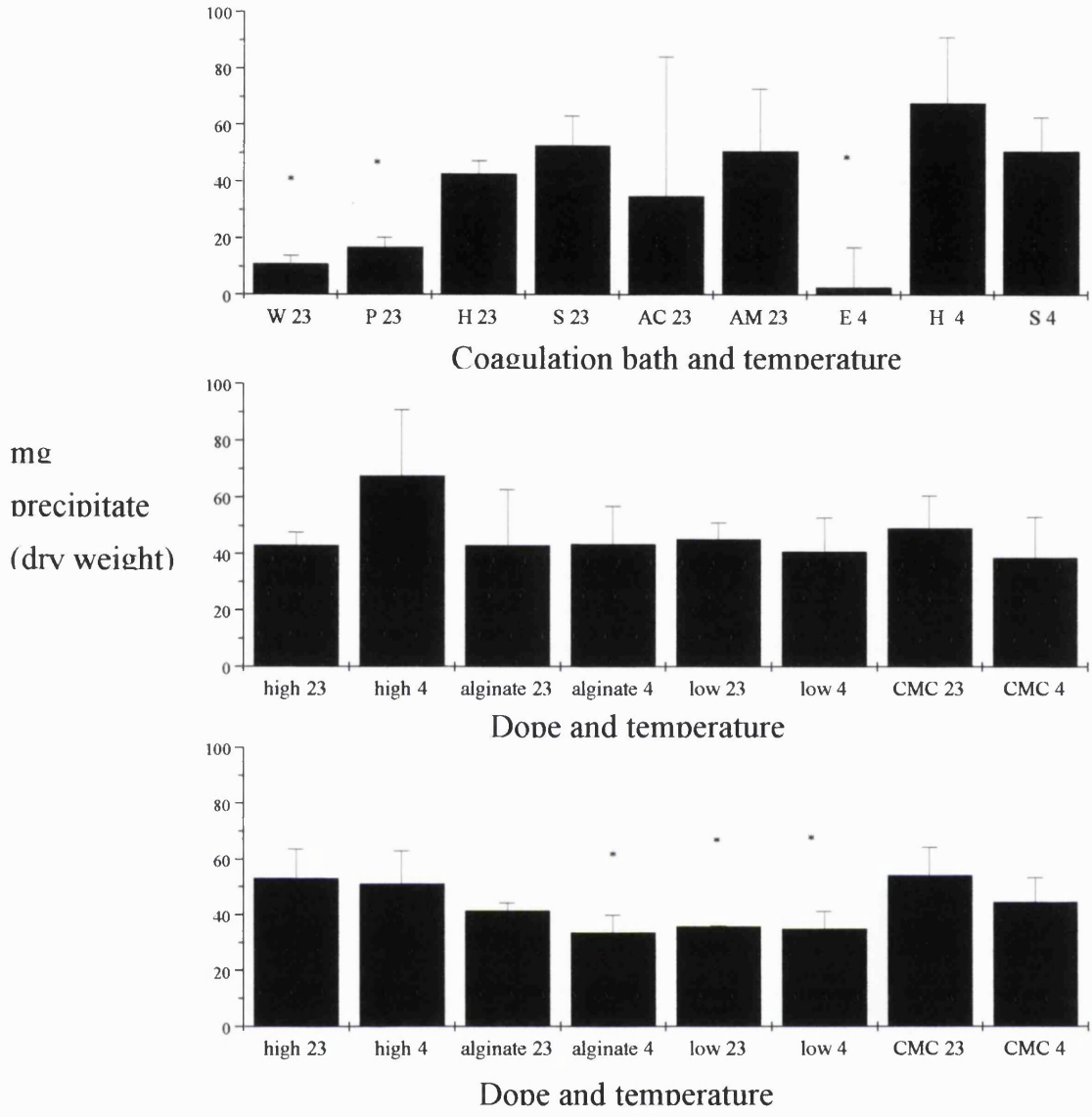


Figure 5.17. *Top: Precipitate (mg) formed when 0.25 mL high concentration (105 mg/mL) protein solution was extruded into a 4 mL of a variety of coagulation baths. Coagulation baths tested were W- water; P - 0.01 M PBS, pH 7.3; H - 0.25 M hydrochloric acid, 2 % $CaCl_2$, pH 0.9; S - 0.05 M sulphuric acid, 2 % Na_2SO_4, pH 1.8; AC - 10 % acetic acid, 10 % NaCl, pH 1.9; AM - 3 M ammonium sulphate, pH 5.6; E - 60 % ethanol, pH 7.1. Coagulation baths were either 23 or 4°C. Results are an average of 3 experiments and error bars represent the 95 % confidence interval for the data. * represents results significantly different at the 95 % level following independent t tests. Middle: mg precipitated formed when different composition dopes are extruded into 0.25 M hydrochloric acid, 2 % $CaCl_2$, pH 0.9 at either 23 or 4°C. High - protein concentration = 105 mg/mL;. alginate - 90 mg/mL + 1% sodium alginate; low - 90 mg/mL with no additive; CMC - 90 mg/mL + 0.5 % CMC. Bottom: As middle graph but extrusion into a 0.05 M sulphuric acid, 2 % Na_2SO_4, pH 1.9 coagulation bath.*

5.4. Fibre production from small scale experiments

The amount and character of the fibres produced on extrusion of the different dopes into a $HCl/CaCl_2$ coagulation bath was measured and compared to results for drawn fibronectin cables. The results are summarised in Table 5.3.

The yield of wet spun fibres with and without additives was greater than the yield of drawn cables, in terms of cm fibre / mL of starting material. However, when cm fibre / mL starting material was transformed into a measure of fibre length / mg protein in starting material, the yield of the wet spun fibres was lower than that for drawn fibronectin cables, due to the high concentration of protein required for wet spinning. It has been calculated that 13m of drawn cable can be produced per litre of plasma donation (see section 4.5.1.). Using this figure the number of metres of wet spun fibre which could be produced per litre of plasma donation are 1, 1.5 and 2.3 metres for fibronectin plus alginate, fibronectin alone and fibronectin plus CMC respectively.

The spinning dopes were extruded through a 0.5 mm diameter needle into the acid. The fibres made from protein alone and protein and CMC stuck to both the coagulation bath and other fibres. These fibres could be drawn to a finer diameter, which may account for the increased yield for these fibres, 138 and 182 cm/mL for fibronectin alone and fibronectin plus CMC compared to 73 cm/mL for fibronectin plus alginate. The alginate containing fibres had a consistent wet diameter, approximately 1mm, but could be drawn if required. The diameter of the fibronectin alone and CMC containing fibres varied according to the degree of drawing.

In Table 5.4, the yield of fibre / mg protein in dope has been combined with approximate diameters for the cables after collection in these experiments. In this case the volume of drawn fibronectin cable produced per mg of protein in dope is similar to the volume of spun fibronectin fibre in the presence of alginate. Therefore drawing the spun fibres down to a finer diameter after formation redresses the imbalance seen in the yield when the high concentration of the protein solution is taken into account. Importantly there is probably less protein wastage during a fibre spinning process than that seen after cable drawing, where 20 % protein remains in solution.

All the spun fibres had a significantly higher moisture content than the drawn fibronectin cables (p=0.000, independent t-test at 95 % level of significance). This is due to their high protein content. This moisture will lead to a low wet tensile strength, commonly associated with protein fibres but aids the drawing of the fibre to the required diameter.

Addition of alginate to the fibres reduced the fibre's tendency to stick to each other and the coagulation bath.

Fibre type	Yield (cm/mL dope) ± 95 % C.I (cm/mg protein in solution)	Wet diameter range (μm)	Fibre character	Moisture content (%) ± 95 % C.I
Spun Fn	137.5 ± 25.1 (1.3cm/mg)	100-1000	easily drawn, self-adherent	88.9 ± 0.7
Spun alginate	73.1 ± 8.6 (0.8cm/mg)	1000	can be drawn, not self-adherent	91.4 ± 1.4
Spun CMC	182.0 ± 32.9 (2.0cm/mg)	200-1000	easily drawn, self-adherent	92.3 ± 0.3
Drawn Fn	52.5 ± 18.9 (11.2cm/mg)	100-500	easily drawn	76.0 ± 1.7

Table 5.3. *Yield and moisture content of wet spun fibronectin fibres compared to drawn cables. All fibres had protein composition 65 % fibronectin / 35 % fibrinogen. Yield was calculated as cm fibres formed on extrusion into a 0.25 M hydrochloric acid, 2 % calcium chloride coagulation bath. Approximate wet diameter was measured and whether the fibres stuck to one another or could be easily drawn was noted. Moisture content was measured by a dry weight method.*

Fibre type	cm/mg protein (from Table 5.3)	Assumed average diameter after drawing, where appropriate (cm)	cm^3 fibre / mg protein in dope ($x10^{-3}$)
Spun Fibronectin	1.3	0.05	2.6
Spun alginate	0.8	0.08	4.0
Spun CMC	2.0	0.05	3.9
Drawn Fibronectin	11.2	0.02	3.5

Table 5.4. *Comparison of the total volume of fibre produced per mg protein in dope. Assumptions have been made about the average diameter of the fibres or cables after drawing. The fibres are presumed to be cylindrical.*

5.5. Altering initial precipitate composition

The proportions of the two proteins, fibronectin and fibrinogen in the spinning dope can be altered, either by manipulation at the PEG precipitation stage or by purification of both proteins and subsequent mixing to produce the required concentrations.

5.5.1. Spinning high fibronectin / high fibrinogen materials

Affinity chromatography purified fibronectin was PEG precipitated, dissolved at a high concentration in 6 M urea and spun into a HCl/ $CaCl_2$ bath. The fibres formed were highly sticky and almost transparent. They could be drawn but were difficult to handle. Fibres have also been spun from a 75 % fibrinogen / 25 % fibronectin dope. These fibres were fairly sticky but easier to handle than the pure fibronectin fibres.

The composition of the fibres could be altered to reflect the final properties of the material required. Fibres made from a high proportion of fibronectin are required for materials promoting good cell adhesion. Section 4.3.2. discusses balancing fibronectin amd fibrinogen concentrations to allow for both cell adhesion and migration. The solubility curves for solutions with different proportions of the two proteins are all similar (section 4.3) and both proteins are denatured to some extent and precipitated at a pH <1.0. Thus a strong acid coagulation bath could be used to spin fibres of varying

protein compositions although the final fibres will have different material and cell adhesion properties.

5.6. Summary

The aim of this section of work was to examine the feasibility of wet spinning fibronectin / fibrinogen fibres and to investigate some of the factors involved in the wet spinning process on a small scale.

Protein concentration: Fibres could be spun on a small scale (extrusion of 1-2 mL) from protein solutions with total protein concentration greater than 70 mg/mL. 6 M urea was used to dissolve the protein / PEG precipitate to this concentration and to open out the protein molecule's structure to allow fibre formation. Other solvents such as guanidine hydrochloride and sodium hydroxide should be examined to see if they too can be used to dissolve fibronectin and fibrinogen. The treatment chosen must avoid the loss of the protein's cell binding ability.

Viscosity: The solution viscosity is crucial to the extrusion and fibre formation process. The viscosity must be high enough to allow the dope to flow from the spinneret in a continuous stream. Increasing the protein concentration led to an increased viscosity, as did chilling the solution to 4-5°C. Solutions with concentration approximately 80 mg/mL and at 4°C started to form gels and were difficult to extrude.

A variety of high viscosity additives could be used to increase the viscosity of the solution and alter the flow behaviour of the solution. The advantage of a shear thinning solution is that the pressure drop over the spinneret will be decreased and thus the pumping requirements are less but the final properties of a fibre formed from a shear thinning dope may depend on the shear to which the solution is exposed at the spinneret. This will differ with spinneret configurations and care must be taken with shear thinning solutions to monitor the viscosity at the point of extrusion. This requires a knowledge of the rheological parameters for the solution at the appropriate temperature and a study of the shear exerted on the solution as it passes through the spinneret.

Addition of 1 % PEO, 1 % sodium alginate and 0.5 % CMC to protein solutions all

resulted in the formation of stable fibres. Addition of alginate reduces the tendency of the protein fibres to stick together.

Coagulation baths: A variety of acid and salt combinations can be used to precipitate the protein. Increasing the salt concentration in the coagulation bath reduces the concentration of acid required. Extrusion into baths with pH < 1.0 produced more stable fibres than extrusion at the pH of minimum solubility, 3.0. The acid will be involved in some denaturation and opening up of the chains allowing them to aggregate into fibres. Cooling the coagulation bath did not make a significant difference to fibre formation. Results for the $HCl/CaCl_2$ bath were more consistent than the H_2SO_4 containing bath.

Yield: The protein concentration required for wet spinning is higher than that for drawing fibronectin cables, (>70 mg/mL compared to 5 mg/mL). Consequently the yield of fibre formed / mg of protein was lower for the wet spun fibres, 1-2 metres per litre of plasma donation compared to 13 metres for the drawn fibronectin cables. However, the wet spun fibres were of a greater diameter than the drawn cables but can also be drawn down to a finer diameter and this redresses the imbalance seen in the above yields.

This chapter described the development of a small scale wet spinning technique. The favoured technique involved extrusion of 1-2 mL of > 70 mg/mL 65 % fibronectin / 35 % fibrinogen into a 0.25 M hydrochloric acid, 2 % calcium chloride bath, pH <1.0, although other combinations were feasible. Chapter 6.0 will focus on the scale up of this technique to produce continuous lengths of fibre.

6.0. Pilot scale production of protein fibres and their properties

6.1. Introduction

A number of protein fibres have been produced for use in the textile industry, for example: casein, soya bean, peanut, corn and groundnut, mainly for the formation of wool blends. Large scale production of these fibres occurred principally between 1937 and 1957, their poor wet strength being one of the reasons that production ceased (Moncrieff, 1969) although interest is still maintained in spinning protein fibres to form textured foods.

Although the manufacture of protein fibres on an industrial scale has been previously carried out, every protein has individual characteristics and properties which present a new challenge for the application of the wet spinning process. This chapter describes a new spinning challenge, the use of a pilot scale textile spinning rig to wet spin fibronectin - fibrinogen fibres for their use in tissue engineering. It was hoped that conditions suitable for spinning from a syringe would be applicable to the pilot scale spinning trial and that continuous extrusion would aid the formation of reproducible fibres.

6.2. Dope preparation and set-up of spinning rig

The rig was set up as shown in Figure 2.2d. The arrangement of holes in the spinneret through which the protein was extruded could be altered and a summary of the tested configurations is given in Table 6.1.

On a small scale, fibres could be wet spun from a solution containing at least 70 mg/mL total protein. However, a 95 mg/mL solution mixed with 1 % sodium alginate and dissolved in 6 M urea did not form stable, continuous fibres when extruded into 0.25 M hydrochloric acid, 2 % calcium chloride, pH < 1.0 through spinneret type 3. On pouring, the solution did not form a continuous thread and on extrusion discrete fibres were not formed. Instead the protein coagulated into white beads. To reduce the problem of capillary breakage it was deemed necessary to increase both the solids content and viscosity of the solution. This was done by either increasing the alginate or the protein concentration of the solution. Further trials were carried out using dopes made up as

described in section 2.3.4.

The formation of coagulated beads was caused by die swell or the 'Barus effect'. As a result of the shearing effect on the fluid in the capillary, on leaving the spinneret the fluid contracted axially and the radius of the fluid jet increased (Ziabicki, 1976). In this case the increase in radius was large, the protein coagulated quickly and there was no time for formation of an elongated fibre. The result was the formation of spheres of coagulated protein. The effect of die swell depends on the shearing history of the molecule (Walczak, 1977).

In Table 6.1. estimates of capillary wall shear and mass average shear rate have been made for each of the spinneret configurations tested using equations 6.1-6.3:

Type	Hole radius, r (m)	Capillary length, L (m)	No. of holes	Flowrate Q (m^3s^{-1})	Capillary wall shear rate, γ_w (s^{-1})	Mass average shear rate γ_{av} (s^{-1})	Residence time T (s)	Total shear γ_{av}*T (-)
1	1.0E-03	0.038	1	2.5E-07	318	170	4.78E-01	81
2	5.0E-04	0.018	1	2.5E-07	2550	1360	5.65E-02	77
3	6.0E-05	0.0001	20	1.3E-08	73700	39300	9.05E-05	3.6
4	1.5E-04	0.0001	8	3.1E-08	11800	6290	7.20E-05	0.5

Table 6.1. *Properties of spinnerets used for fibre production. An average flowrate used during the spinning trials of 15 mL/min was assumed here. Wall and mass average shear rates and capillary residence times were calculated from equations 6.1 - 6.3 given below. For comparative purposes mass average shear rate and residence time were multiplied to give a measure of total shear in the spinneret. Laminar flow was assumed.*

$$\gamma_w = \frac{4Q}{\pi r^3} \qquad \text{(equation 6.1)}$$

$$\gamma_{av} = \frac{8}{15}\gamma_w \qquad \text{(equation 6.2)} \qquad \text{(Bell, 1982)}$$

$$T = \frac{L\pi r^2}{Q} \qquad \text{(equation 6.3)}$$

The mass average shear rate calculated for spinneret 3 was very high, 7.9 x 10^5 s^{-1}. Solutions containing alginate have shear thinning properties and consequently have a low apparent viscosity at a high shear rate. This low viscosity reduces the solution's ability to form a continuous stream of flow and does not aid fibre formation.

From equation 6.1, γ_w is proportional to $\frac{1}{r^3}$, thus very small spinneret holes result in a large shearing effect on the fluid. By increasing the diameter of the spinneret hole to 1 or 2mm, the mass average shear rate was reduced to 1358 and 170s^{-1} respectively. Shear in a capillary is believed to align molecules in a fluid, in preparation for extrusion and coagulation. The time over which the solution was exposed to these lower shear rates was increased by using long capillaries (18 and 38 mm). A calculation of total shear was made as a theoretical measure of both the shear rate, due to the spinneret hole size or capillary diameter, and flowrate and the residence time in the capillary. Higher values of total shear, 81 and 77, were obtained for spinnerets 1 and 2 than for the small holed spinnerets, 3 and 4. However these higher total shear values combined a relatively low average shear rate and a long residence time to align the protein molecules before extrusion and reduce the effect of die swell on entry into the coagulation bath. In order to spin fibres through the very small holed spinnerets it is essential that solutions have a high viscosity at high shear rates so that continuous fluid flow is maintained. This would necessitate a study of the rheological parameters of the chosen spinning dope.

The viscosity of three potential spinning dopes was measured and the rheogram Figure.6.1. used to investigate the solutions' flow behaviour. The 98 mg/mL protein solution, dissolved in 6 M urea, exhibited Newtonian behaviour between shear rates 30 - 1000s^{-1} and showed no thixotropic or rheopectic behaviour. The shear rate in capillary 2 was calculated as 1358 s^{-1} for a flowrate of 15 mL/min. As an approximation to the viscosity of the solution in the capillary, the viscosity at a shear rate of 1000s^{-1}, when measured in the viscometer, was found to be 55 mPa s (0.55 Poise). Dope containing 140 mg/mL protein and 1.3 % alginate in a 6 M urea solution (high protein) formed a shear thinning solution (n = 0.76 for shear rates 7 - 1000s^{-1}). No rheopectic or thixotropic behaviour was seen and the apparent viscosity at 1000s^{-1} was 413 mPa s (4.1 P).

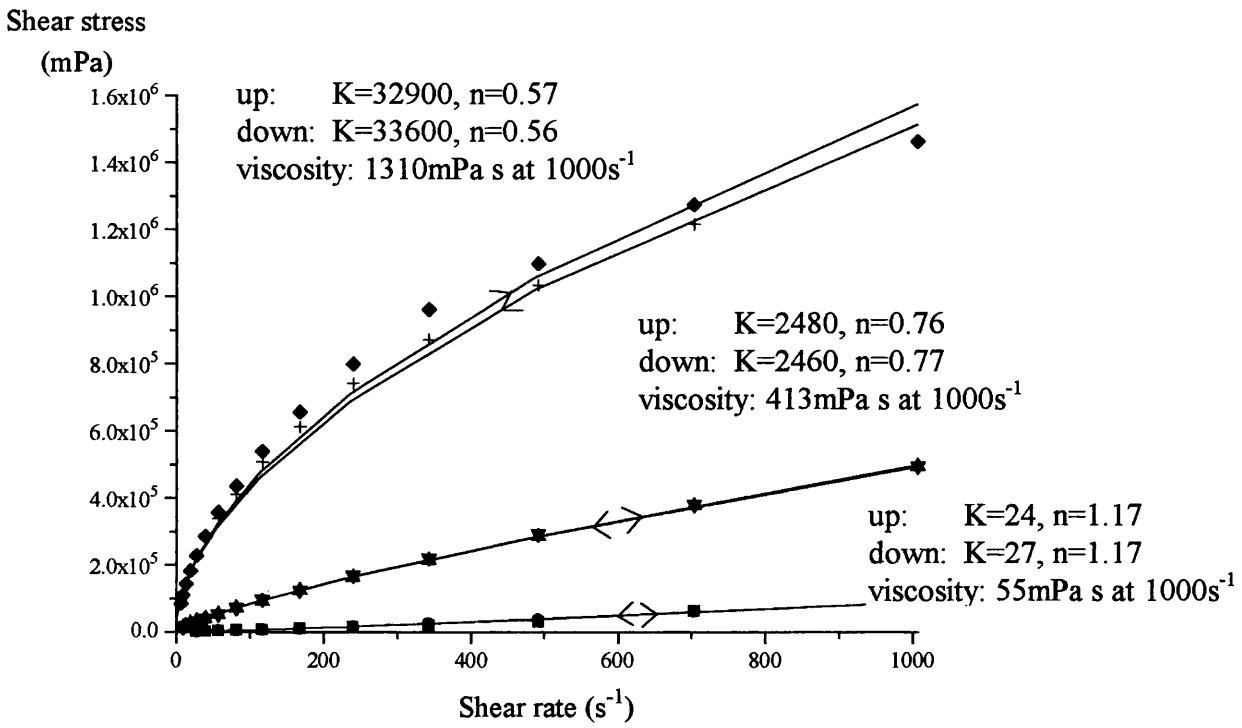


Figure 6.1. *Shear rate versus shear stress for different spinning dopes.◆ - 46 mg/mL protein, 4.8 % alginate, up curve; + - down; ▼ - 140 mg/mL protein, 1.3 % alginate, up; ▲ - down; ■ - 98 mg/mL, up curve; ● - down. Power law consistency index, K (mPa s^n), and exponent, n (-), are given for each set of curves. Apparent viscosity is given for shear rate 1000s^{-1}. Measurements were made up to a shear rate of 877s^{-1} for the 98 mg/mL protein only solution and so the viscosity given assumes Newtonian behaviour at 1000s^{-1}. All measurements were made on freshly made solutions at 23°C.*

To increase the viscosity further, the alginate concentration was increased to 4.8 % and the protein concentration decreased to 46 mg/mL (high alginate). This produced a highly viscous, shear thinning solution, with power law exponent n = 0.57 (shear rates 7 - 1000s^{-1}) and an apparent viscosity of 13.1 P at 1000s^{-1}. Fluid flow behaviour was seen to be slightly thixotropic.

Increasing the concentration of alginate in the solution both increased the solution viscosity and the shear thinning properties of the solution. Table 6.2 gives the power law parameters for 6 M urea, 1 % and 4 % alginate solutions with no protein present.

Increasing the solids content and increasing the alginate concentration enhances the spinning viscosity of the solution. Young and Lawrie (1974) quote a recommended range of viscosity for spinning as 25 - 380 Poise and have spun bovine plasma proteins with a viscosity of 250 Poise (spinneret diameter 8 μm). They have also spun porcine lung and

stomach proteins with apparent viscosities of 2 - 7 Poise at a shear rate of $517s^{-1}$(Young and Lawrie, 1975a).

Solution	Curve	K ($mPa\ s^n$)	n (-)	Apparent viscosity at $1000s^{-1}$ (mPa s)	Shear rate range (s^{-1})
6 M urea	up	1.4	0.98	1.23	100-877
6 M urea	down	1.4	0.99	1.23	100-877
1 % sodium alginate	up	53.1	0.87	22.5	24-3680
1 % sodium alginate	down	50.9	0.88	22.5	24-3680
4 % sodium alginate	up	3037.7	0.73	388.0	7-1007
4 % sodium alginate	down	2959.3	0.73	388.0	7-1007

Table 6.2. *Rheological properties of solvents and additives without fibronectin. Consistency index and power law exponent are given for 6 M urea, 1 % and 4 % sodium alginate dissolved in 6 M urea. All measurements were made at 23°C. Shear rate range depends upon measuring device used and solution viscosity. Measurements for a 6 M urea solution were made up to a shear rate of $877s^{-1}$. Newtonian behaviour has been presumed to give a viscosity at $1000s^{-1}$.*

Extrusion of 140 mg/mL protein /1.3 % alginate solution through spinneret 1.

Continuous fibres were formed on extrusion through the 2 mm diameter spinneret into 0.25 M HCl, 2 % $CaCl_2$, pH 0.9. However fibres only approximately 25 cm long could be spun without breakage. This was due to the thickness of the fibre causing poor diffusion of acid into the fibre's core, preventing total coagulation of the protein. To reduce this effect spinneret 2, with an orifice diameter of 1 mm, was used.

Extrusion of 140 mg/mL protein / 1.3 % alginate solution through spinneret 2.

The fibres formed on extrusion into 0.25 M HCl, 2 % $CaCl_2$ were several metres long and could be collected on rollers. The fibre diameter was decreased by setting the rotational speed of the collecting roller to turn faster than the drawing roller (Figure 2.2d). Speeds chosen were 31 and 21 rev./min. respectively.

Wet spun fibres are difficult to collect on rollers because of their high degree of

hydration. Fibronectin / fibrinogen drawn cables had been found previously to be highly hygroscopic (see section 4.5.4.) and this posed a collection problem. To remove some of the water in the fibres, they were passed through a 100 % acetone bath after washing. 100 % acetone was used so that the solvent evaporated quickly and reduced the time during which the protein could be denatured. Walczak (1977) suggested centrifugation as a method of removing liquid from highly hydrated fibres without denaturing the protein. Other fibres were washed and not dehydrated, their properties are compared in section 6.3. Fibres could also be spun through this spinneret into a 10 % sulphuric acid bath, which is often used effectively to both coagulate and dehydrate fibres.

Some 140 mg/mL protein / 1.3 % alginate fibres were transparent when drawn, indicating good orientation. Others were white and opaque, caused by light refracting within the fibre, an indication of poor internal orientation.

Extrusion of 46 mg/mL protein / 4.8 % alginate through spinneret 2.
Fibres spun from this solution contained numerous air bubbles, indicating that the dope had not totally degassed overnight, however, several metres of fibre could be spun continuously. The fibres produced appeared both porous and white. The wet strength of the material was poor and no decrease in diameter could be made by drawing. Consequently both collecting and drawing roller speeds were set at 16 rev./min.

Extrusion of 46 mg/mL protein / 4.8 % alginate fibres spun through spinneret 4
The fibres formed were weak, did not stick together, but dried quickly onto the rollers and could not be removed easily. Only small lengths (5-10 cm) of these fibres could be spun continuously. It has been estimated that the spinneret orifice diameter should be twice the final fibre diameter required and thus the final fibres produced after extrusion through spinneret 4 had a diameter less than 100 μm. Since these fine fibres were difficult to handle, extrusion through the 1 mm diameter spinneret was preferred.

The aim was to produce some protein dopes which could be used to spin fibres on a pilot scale. One problem with dissolving protein precipitate directly into urea to give a high concentration protein solution was the initial water content of the precipitate. This diluted the 6 M urea and reduced its effective solvating power. Concentrated urea could potentially act as a virus inactivation step in the process (see chapter 8.0) so this dilution

effect must be considered. Methods of avoiding this solvent dilution could include freeze drying the protein precipitate before addition to the solvent or addition of solid 6 M urea to the precipitate (see section 6.4). An ideal spinning dope was considered as a clear, viscous solution with a high protein content, which could be poured in a continuous stream.

Fibres made from both high alginate and high protein dopes when spun through spinneret 2 (diameter 1 mm) were collected, with and without acetone dehydration and their properties tested.

6.3. Properties of wet-spun fibres

The general appearance of acetone dried, wet spun fibres is shown for both high alginate and high protein fibres in Figures 6.2 and 6.3 respectively. The surface of the high alginate fibres consists of clearly visible ridges, approximately 10 μm wide, aligned along the longitudinal axis. Ridges are also present on the surface of the high protein fibres, but can only be seen at a higher magnification and have an estimated width of 1 μm.

6.3.1. Tensile properties

Tensile properties of the material were examined by the measurement of ultimate tensile strength, set and extension at break. Favourable properties for large scale processing were considered as high tensile strength, extension at break > 10 % and evidence of set. Set is the permanent increase in fibre length after tensile testing and would allow a fibre to be drawn to a finer diameter when dry. The tensile properties varied with the fibre's composition, Table 6.3. Fibres were also tested at 2 ranges of relative humidity and an examination of water absorption properties of drawn cables demonstrated hygroscopic behaviour. Theoretically, if the relative humidity of the testing atmosphere is altered, the amount of water absorbed by the fibre will also change, affecting the fibre's tensile properties. A sample stress-strain profile obtained during tensile strength measurement of these fibres is shown in Figure 6.4.

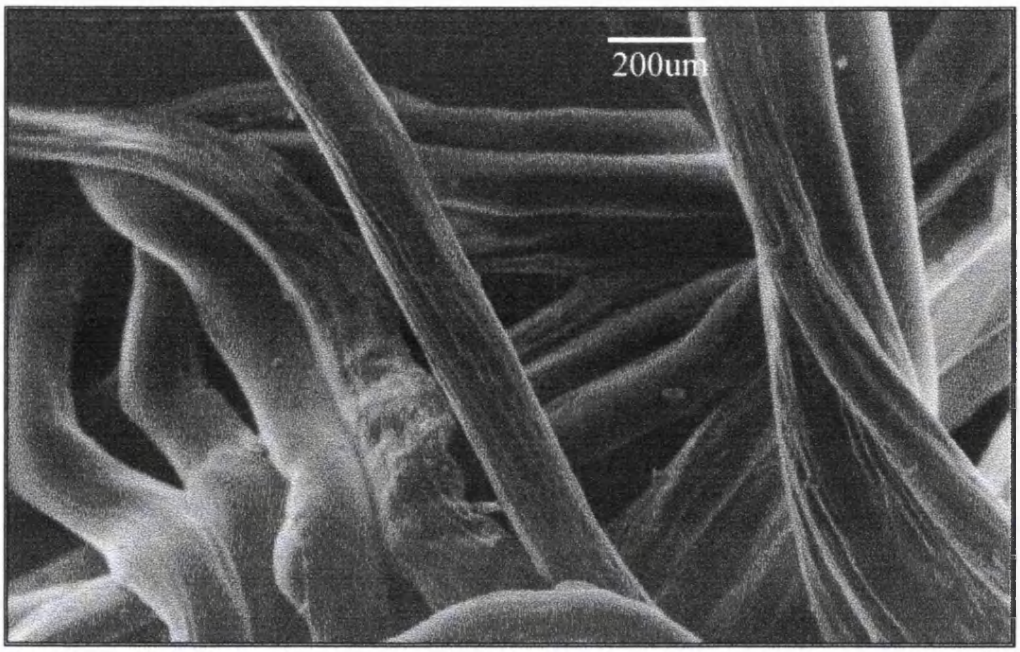


Figure 6.2. *Scanning electron micrograph of surface view of 46 mg/mL protein / 4.8 % alginate fibres (60 % fibronectin / 40 % fibrinogen) spun on a small scale using a syringe but made from one of the dopes tested at pilot scale. Surfaces ridges are visible running parallel to the long axis of the fibre. Higher magnification micrographs showed a number of minor ridges , approximately 10 μm wide.*

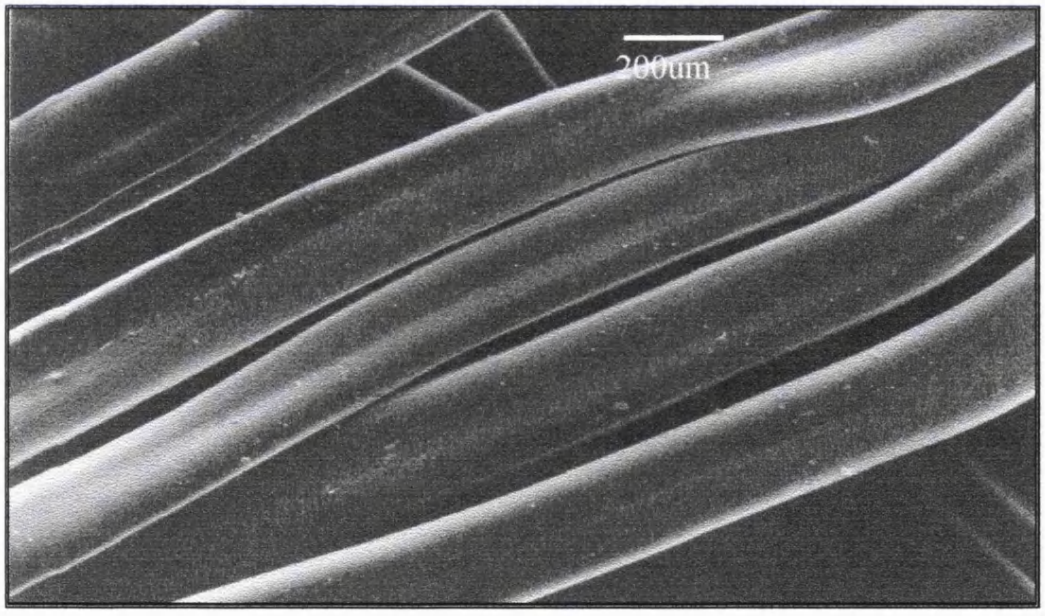


Figure 6.3. *Surface view of 140 mg/mL protein / 1.3 % alginate fibres (60 % fibronectin / 40 % fibrinogen) spun from a syringe using one of the dope compositions tested at a pilot scale. At this magnification the surface of the fibres appears smooth. However at a higher magnification, small ridges approximately one micron wide and running parallel to the long axis of the fibre can be seen.*

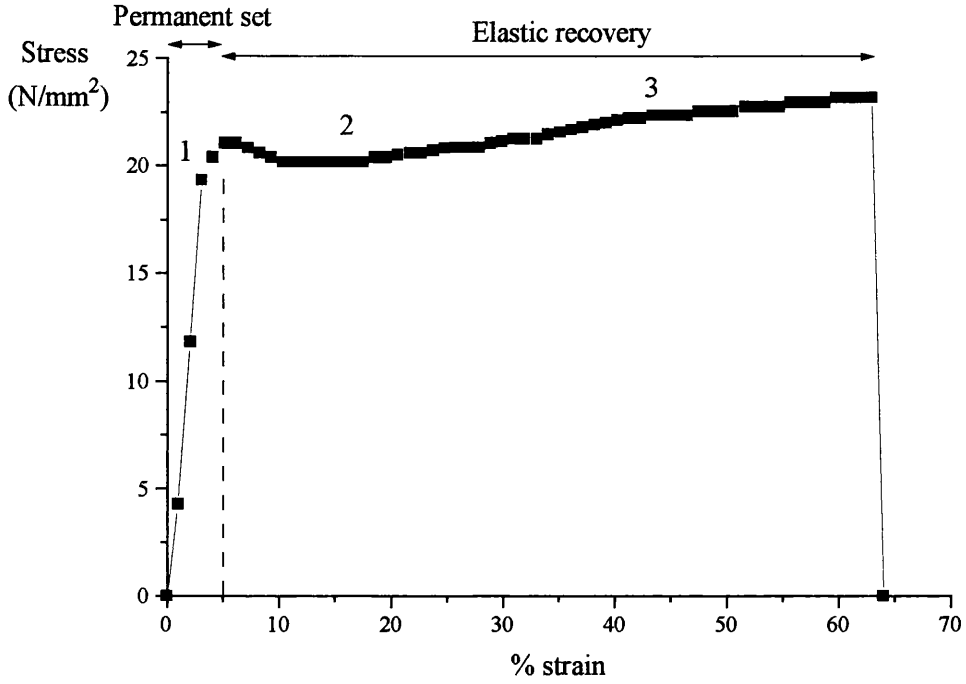


Figure 6.4. *Typical stress -strain curve for wet spun fibres. Stress is calculated as load applied per unit area and expressed in N/mm^2. Strain is expressed as an increase in the original length of the cable. The ultimate tensile strength of the cable here is 23N/mm^2. The dashed line shows the border of the elastic (left side) and plastic regions (right side).*

The only similarity between this stress-strain profile and that for brittle, drawn cables, Figure 4.22, is an elastic region where the stress is proportional to the strain (marked 1). Under this strain, if the load was removed then the sample returned to its original length, hence the name elastic region. However for the wet-spun fibres if an extra load was applied then the fibre exhibited partially elastic behaviour. Removal of the load resulted in recoil of the fibre, elastic recovery, but there was also a permanent increase in length, termed permanent set (Gere and Timoshenko, 1991). This set was not a feature of the brittle cables and represented a change in the material's internal structure.

In the plastic region (marked 2), the protein molecules increased their degree of alignment and improved the crystalline structure of the fibre. The fibre diameter also decreased. Shortly before fracture the slope of the stress-strain curve started to rise (marked 3), indicating strain hardening. This indicated further changes in internal fibre structure and increased resistance to further deformation.

Protein conc. (%)	Alginate conc. (%)	Temp.(°C) RH (%)	Tensile strength (N/mm^2)	Set (%)	Elongation at break (%)	Acetone dehydrated
4.6	4.8	24 - 25 58 - 69	6.6 ± 1.6 (11)	4.9 ± 3.5 (11)	21.4 ± 6.1 (11)	yes
4.6	4.8	24 - 25 59 - 67	1.7 ± 0.3 (9)	1.4 ± 1.8 (6)	18.6 ± 7.2 (9)	no
14.0	1.3	24 - 25 59 - 67	28.1 ± 9.2 (11)	20.5 ± 6.5 (11)	52.4 ± 14.3 (11)	yes
14.0	1.3	24 - 25 60 - 70	7.5 ± 2.0 (9)	11.8 ± 7.2 (8)	43.1 ± 11.8 (9)	no
4.6	4.8	23 - 24 39 - 49	9.3 ± 1.5 (12)	7.4 ± 3.1 (10)	20.2 ± 7.3 (12)	yes
14.0	1.3	23 - 24 41 - 50	21.3 ± 5.3 (12)	18.7 ± 5.9 (10)	39.0 ± 11.2 (12)	yes

Table 6.3. *Tensile properties of wet spun fibronectin / fibrinogen fibres. Samples were tested at either 60-70 % or 40-50 % relative humidity (RH) and at room temperature. Tensile strength was measured as the maximum load required to fracture the specimen per unit cross sectional area. Set is the permanent increase in sample length following breakage, expressed as a percentage of the original length. Elongation at break is the % increase in sample length at the time of fracture, expressed as a % of the original length. Number of samples tested for each group is given in brackets and all results are ± 95 % confidence intervals.*

Statistical tests on the tensile strength data

A simple factorial 2 way ANOVA (analysis of variance) was used to investigate any interactions between humidity and sample type with respect to strength, set and elongation at break. Analysis showed that there was a significant difference in strength, set and elongation at break ($p=0.000$) between the four fibre types: 46 mg/mL protein / 4.8 % alginate with and without acetone and 140 mg/mL protein / 1.3 % alginate with and without acetone. All statistics were performed at the 95 % level of significance. 2 way interactions between sample type and relative humidity for strength, set and elongation at break were non-significant.

An independent t-test was used to establish whether the 2 different compositions or treatments had any effect on the fibre properties. The combinations tested were:

1. 46 mg/mL protein / 4.8 % alginate, with and without acetone
2. 140 mg/mL protein / 1.3 % alginate, with and without acetone
3. 46 mg/ml protein / 4.8 % alginate with acetone, 140 mg/mL protein / 1.3 % alginate, with acetone
4. 46 mg/mL protein / 4.8 % alginate without acetone, 140 mg/mL protein / 1.3 % alginate, without acetone

The tensile strength was found to be significantly different for comparisons between all groups ($p=0.001$). For measurement of set, treatment with acetone did not give a significant difference for either fibre composition (tests 1 and 2) but set was significantly greater for the 140 mg/mL protein fibres in both tests 3 and 4, $p= 0.000$ and 0.014 respectively. The same was true for elongation at break, with p values of 0.520, 0.291, 0.001 and 0.001 for tests 1 to 4.

Fibres made from 140 mg/mL protein and only 1.3 % alginate were significantly stronger than those made from less protein and more alginate. Removal of excess water with acetone from these high protein fibres gave an average tensile strength of 28.1 N/mm^2, compared to 7.5 N/mm^2 with no acetone treatment. Removal of water increases hydrogen bonding and hydrophobic attractions between protein molecules of the fibre and results in increased strength. Increasing the proportion of alginate in the fibre decreases the intermolecular protein bonding and results in a decrease in strength to 6.6. and 1.7 N/mm^2 with and without acetone treatment respectively.

A tensile strength of 28.1 N/mm^2 compares favourably with drawn, non-twisted fibronectin:fibrinogen cables with a tensile strength of 14.9 N/mm^2. The twisted cables with increased intermolecular bonding were stronger with an average strength of 60.9 N/mm^2 (see Table 4.1).

Berger *et al.*, (1996) quote the tensile strength of other biological polymers. Tendon collagen has a tensile strength of 75 N/mm^2 (75 MPa) whereas keratin and costal cartilage have tensile strengths of 50 and 1 N/mm^2 respectively. Collagen with its high tensile strength is the main structural protein in the body, whilst keratin is the main

protein in hair and nails and is highly cross-linked. Cartilage, with a low tensile strength, is a highly hydrated tissue providing the bearing surfaces in joints. Twisted fibronectin cables have a tensile strength of the same order of magnitude as tendon collagen and keratin but are extremely brittle. The wet spun fibres do not have this high tensile strength for load bearing functions but have improved flexibility when dried.

Set represents the permanent increase in length of the fibre following tensile strength testing. The high protein fibres showed greater permanent deformation than those with a greater proportion of alginate, 20.5 % and 11.8 % compared to 4.9 and 1.4 % depending on acetone treatment. The protein molecules in the fibres will again be in a linear, extended form. As the fibre is drawn, the molecules will be pulled past one another and can make new bonds with different molecules giving a permanent increase in length. Increasing the proportion of alginate breaks up this linear arrangement of molecules and reduces the number of successful inter-protein interactions. Extension at break is also greater for the high protein fibres, 52.4 and 43.1 % compared to 21.4 and 18.6 % for the 46 mg/mL protein / 4.8 % alginate fibres. Again the linear arrangement of the protein molecules allows chains of the molecules to pass each other easily and increase the length of the fibre. Acetone dehydration does not affect this process. Elongation at break for drawn protein cables was previously found to be <5 %, causing handling problems. The elongation at break for these wet spun fibres will make them easier to draw down to finer diameters and collect on mechanical rollers, however their relatively low wet and dry tensile strengths, particularly of the high alginate fibres, may pose a problem.

The fibre's capacity to bend (flexibility) was measured by wrapping samples around steel rods of decreasing diameter. Wet spun fibres could be wrapped around all the rods in a tight spiral motion at least 5 times down to a 0.0625 inch diameter whereas the drawn protein cables could not be wrapped around the 0.25 inch diameter rod. The tensile properties of the wet spun fibres appear to have significant advantages over the drawn cables for consideration of process design.

The results were expected to show a decrease in tensile strength with increasing relative humidity. Although no two-way interactions were found between strength, set and elongation at break with the 2 levels of humidity and fibre composition, the independent t-test showed that the increase in strength at 40 % relative humidity was significant,

compared to 60 % relative humidity, (p=0.014) for the dehydrated 46 mg/mL protein / 4.8 % alginate fibres. The hysteresis moisture adsorption curve for the protein material may have been altered by the addition of alginate so that more water is absorbed at 60 % relative humidity by these 'high' alginate fibres than by the high protein fibres. This led to a decrease in tensile strength at 60-70 % relative humidity compared to the 40-50 % test, 6.6 and 9.3 N/mm^2 respectively. The incubation time for the high protein fibres at the higher humidity level may not have been long enough for an equilibrium state to be reached. In general all protein fibres show poor wet strength (Moncrieff, 1969).

6.3.2. Diameter and fibre swelling

Fibres were incubated in Dulbecco's modified eagles medium (DMEM) at 37°C for four hours and the increase in their transverse dimensions measured. For both fibre compositions the acetone dehydrated fibres showed a greater increase in diameter than the non-dehydrated fibres. A summary of results is given in Table 6.4. Values of 140 % compared to 96 % and 110 % compared to 98 % were seen for the high protein and high alginate fibres respectively with and without acetone treatment respectively. Only the high protein fibres with and without acetone showed a significant difference in their degree of swelling at the 95 % level of confidence (p=0.008).

Protein conc. (%)	Alginate conc. (%)	Acetone dehydrated	% increase in diameter at maximum swelling	Average diameter (μm)	Denier (g/9000m)	Tenacity (g/denier)
4.6	4.8	yes	110.2 ± 15.7	415 ± 36	1700.4 ± 180.3	0.054
4.6	4.8	no	98.1 ± 13.4	635 ± 17	3269.4 ± 182.2	0.017
14.0	1.3	yes	139.6 ± 29.2	540 ± 63	2150.2 ± 417.5	0.305
14.0	1.3	no	95.9 ± 15.2	563 ± 33	3520.7 ± 1010.4	0.054

Table 6.4. *Dimensions and tenacity of wet spun fibres. The percentage increase in original fibre diameter on incubation in DMEM at 37°C for four hours was calculated for at least 10 samples from each group. The average diameter of the fibres was measured microscopically for 10 fibres. At least 2 metres of dried fibre spun from the 1mm diameter orifice spinneret was used for the denier calculation. Tenacity was calculated from the average tensile strength, diameter and denier for each group. Results are given ± 95 % confidence intervals.*

Although fibres were spun from a 1 mm diameter spinneret, the actual fibre diameters after drawing (high protein fibres only) and drying ranged from 415 - 635 μm. As expected, the acetone dehydrated fibres were of a smaller diameter than those not treated. The independent pairs t-test comparing high alginate fibres with and without acetone(test 1) and both high protein and high alginate fibres treated with acetone(test 3) showed a significance in diameter, p= 0.000 and 0.003 respectively.

The possibility that the presence of alginate in the material alters the moisture absorption hysteresis curve was discussed earlier. For the high alginate fibres, where the diameter was not decreased by drawing the fibre as they were collected, the dehydrated fibres have a small diameter compared to the high protein fibres. This may be due to more efficient solvent removal by the acetone in the presence of alginate. The high alginate fibres were swollen when first spun, suggesting that the hysteresis curve is altered so that the fibres take up and lose water more easily than the high protein fibres. However the high protein fibres showed a larger increase in diameter on incubation with a physiological strength solution.

Differences in the cross-sectional profile of high alginate and high protein fibres can be seen in Figures 6.5 and 6.6 respectively. Voids formed when air was trapped in the coagulating fibre are seen in the high alginate fibre, which has a circular cross-section. These voids are an indicator of poor solution degassing and reduced fibre strength. In contrast, the non-circular, solid cross-section of a high protein, acetone dehydrated fibre can be seen in Figure 6.6.

The non-uniform appearance of the fibre's cross-section could be caused by the rapid removal of solvent from the fibre surface. If the water/urea was removed more quickly from the fibre surface than it could diffuse from the centre of the fibre to the surface, then a solid protein coat would be formed around the outer surface of the fibre, leaving a 'wet' core. It then becomes difficult to remove the trapped solvent through the solidified protein layer. Solvent will be lost through the outer layer by osmosis and the outer surface will become creased as it becomes too large to surround the core. The formation of a non-uniform cross-section may also be caused by the acid coagulating action being too fast (Walczak, 1977). In this case, the production conditions may be

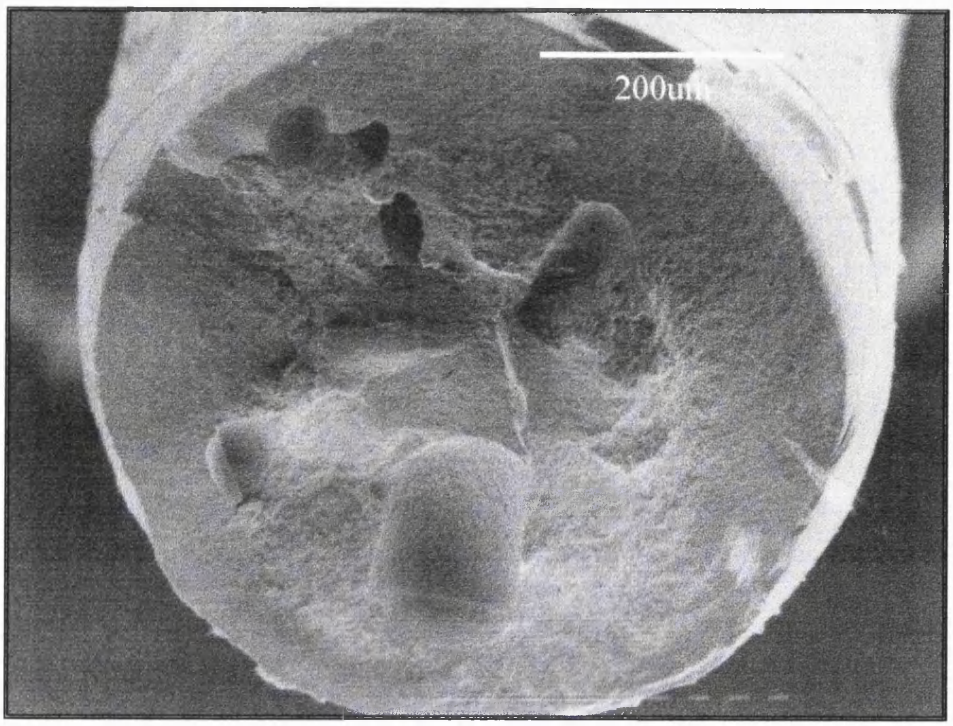


Figure 6.5. *Scanning electron micrograph of the end of a 46 mg/mL protein / 4.8 % alginate fibre spun on the pilot scale rig and treated with acetone to remove excess water. The fibre's cross section is almost circular but contains voids, where air was trapped during the coagulation process.*

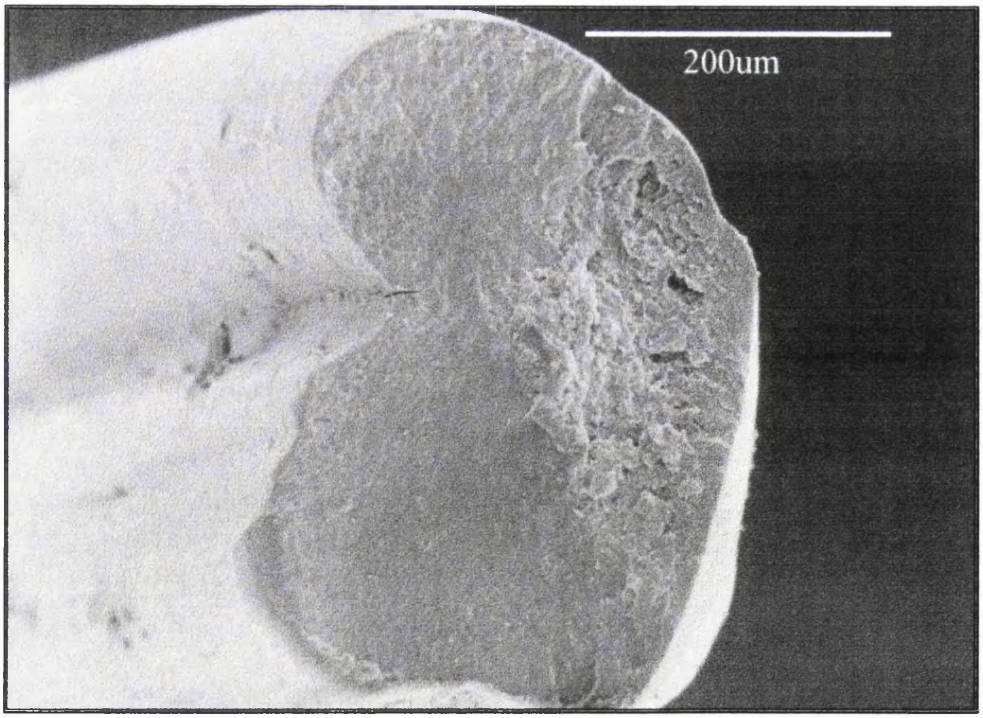


Figure 6.6. *Scanning electron micrograph of the end view of a 140 mg/mL protein / 1.3 % alginate fibre spun on the pilot scale rig and treated with acetone. These fibres were found to have a non-circular cross-section but were solid, leading to fibres with a higher tensile strength when compared to the high alginate fibres.*

more suitable for the formation of the high alginate fibres than the high protein fibres. Using a lower strength coagulation bath would reduce the speed of coagulation and perhaps prevent this buckling of the fibre surface coat.

6.3.3. Denier and tenacity

All fibres had a much higher denier than the drawn fibronectin cables (65 denier). As expected, acetone dehydrated fibres had a lower denier than the non-dehydrated fibres because of their smaller diameter and lower mass. The 46 mg/mL protein / 4.8 % alginate fibres had the lowest denier because they contained numerous air bubbles left over from inefficient degassing of the dope.

The calculated tenacity for drawn, twisted cables was 2.5-3.5 g/denier and 0.7 g/denier for non-twisted cables (see Table 4.2). Tenacity for other protein fibres has been measured as 0.8, 0.8 - 1.0 and 1.2 g/denier for soybean, casein and maize respectively (Moncrieff, 1969). Values for the wet spun fibronectin / fibrinogen fibres are much lower than this. Pure 8-9 % alginate fibres have a low wet strength and have physical properties which vary with moisture content. The tenacity and elongation at break for alginate fibres when tested in a 65 % relative humidity atmosphere was 1.1 g/denier and 14 % (Moncrieff, 1969). The tenacity of the high alginate fibres is compromised by the inclusion of air bubbles in the fibre. Combinations of protein and alginate do not appear to result in a stronger fibre, rather a weaker one.

6.4. Storage of spinning dopes

Young and Lawrie (1974) used both sodium hydroxide and acetic acid to stabilise the viscosity of their bovine plasma protein dopes. They noted a 50 Poise increase in viscosity 15 minutes after the addition of alkali. Their work with porcine stomach and lung dopes (Young and Lawrie, 1975) demonstrated an increase in lung protein isolate viscosity 10 minutes after the addition of alkali and for stomach protein isolates 1-2 hour after the pH was increased.

To reduce the number of additives in the fibronectin / fibrinogen spinning dope, no alkali was used to stabilise the solution viscosity. After mixing, to dissolve the protein precipitate, the solutions contained a large amount of air. To enhance the degassing of

the spinning dopes, they were left overnight at room temperature. This section investigates the storage of spinning dopes both overnight and for long term applications. Tests for stability over long term storage were based on the possibility of making spinning dope from cryoprecipitate in one continuous process and then storing it until required for fibre production.

Viscosity measurements were made when the dope was freshly prepared and at 1, 2, 3, 7 and 8 days after preparation for both 46 mg/mL protein / 4.8 % alginate and 140 mg/mL protein / 1.3 % alginate spinning dopes. Samples were stored at either 23°C or 4°C. On thawing of dope frozen at -20°C, a gel formed which had poor flow properties. Figures 6.7 - 10. show the change in viscosity of the solution and the effect of storage time and temperature on the power law parameters, K and n.

The 46 mg/mL protein / 4.8 % alginate and 140 mg/mL protein / 1.3 % alginate spinning dopes were both shear thinning solutions between shear rates 7 - 1000s^{-1}. From Figure 6.7 the 46 mg/mL protein / 4.8 % alginate dope showed a decrease in apparent viscosity after 8 days storage at 23°C or 3 days storage at 4°C. Figure 6.8. shows changes in the consistency index, K, and the power law index, n, with time and at the two storage temperatures. Within the first 24 hours the consistency index for the dopes stored at both 23°C and 4°C decreased, whilst the n value rose. This represents a decrease in both apparent viscosity and shear thinning properties. Figure 6.7 shows that between shear rates 7 and 500s^{-1} the apparent viscosity for the initial solution and those stored for 1 day at either 23 or 4°C was greater than for solutions stored for a longer period of time. Viscosity at higher shear rates (>500s^{-1}) was less affected by storage time. Solutions still maintained shear thinning properties after storage with n = 0.70 after 8 days storage at 23°C, compared to 0.54 for a freshly prepared solution.

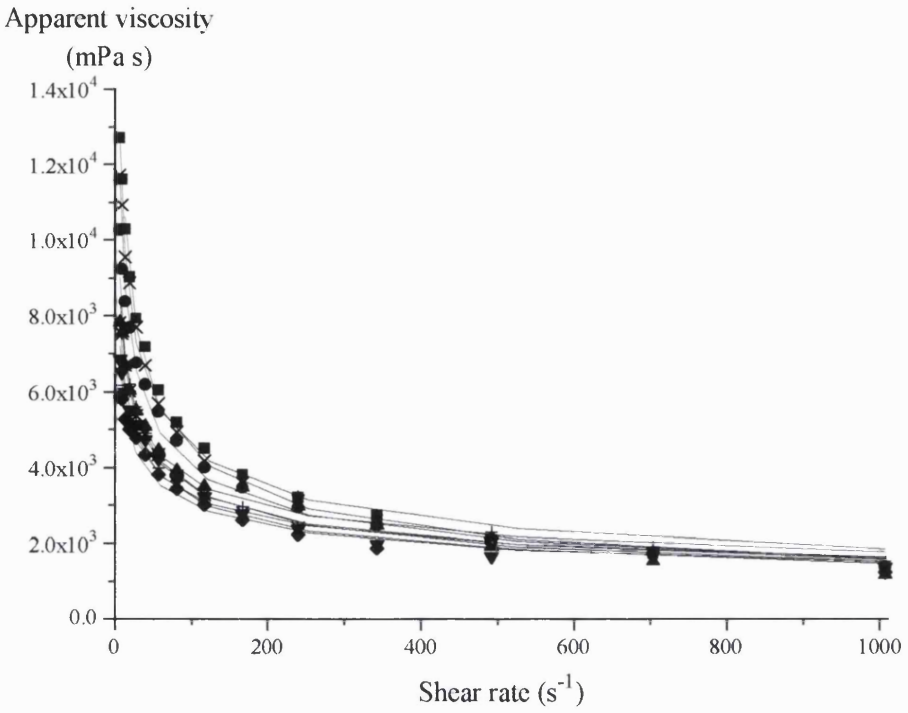


Figure 6.7. *Shear rate vs apparent viscosity for a 46 mg/mL protein / 4.8 % alginate mixture, stored for 8 days at 23°C or 3 days at 4°C.* ■ *- freshly prepared solution; Solutions after storage,* ● *- 1 day, 23°C;* ▲ *- 1 day, 4°C;* ▼ *- 2 days, 23°C;* ◆ *- 2 days, 4°C; + - 3 days, 23°C;* × *- 3 days, 4°C;* ✳ *- 7 days, 23°C; _ - 8 days, 23°C. Curves are fitted using equation 5.3 ($\mu_a = K\dot{\gamma}^{n-1}$).*

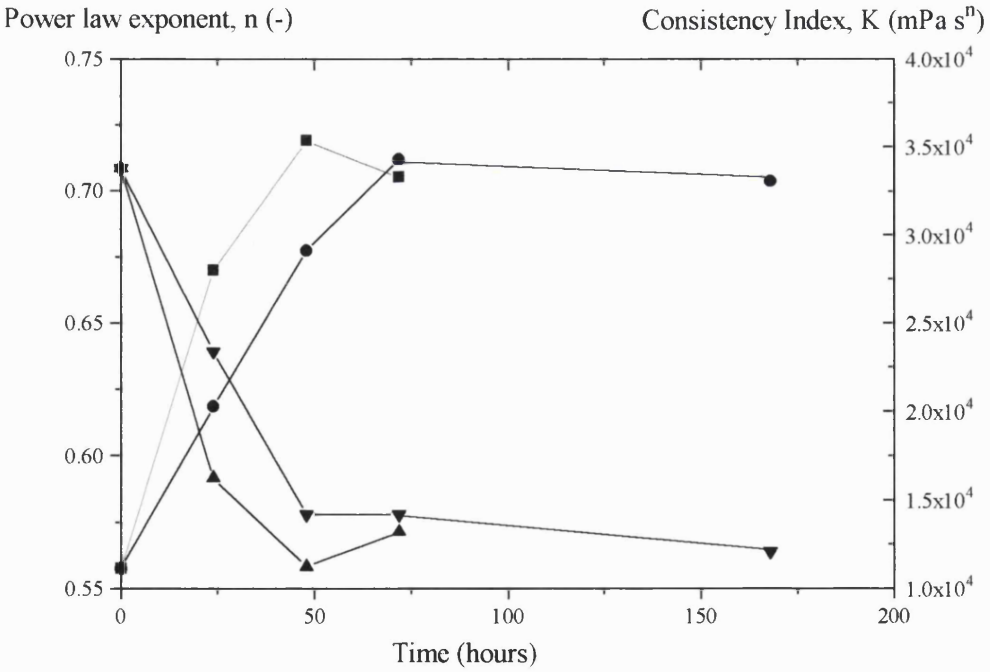


Figure 6.8. *Values of K (mPa s^n) and n (-) versus storage time for 46 mg/mL protein / 4.8 % alginate*
■ *- n, 4°C;* ● *- n, 23°C;* ▲ *- K, 4°C;* ▼ *- K, 23°C.*

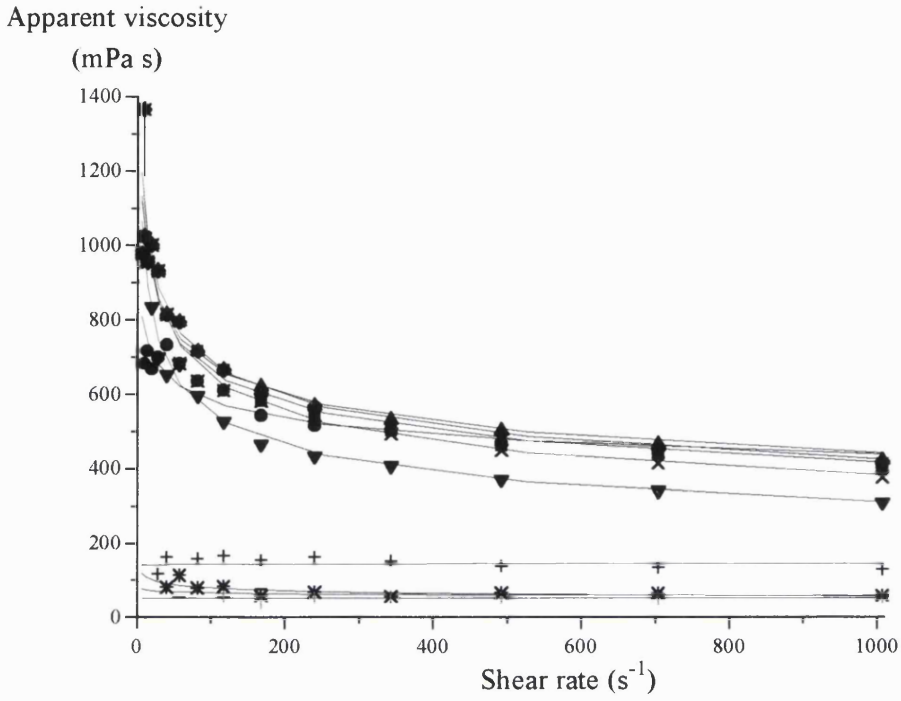


Figure 6.9. *Shear rate vs apparent viscosity for 140 mg/mL protein / 1.3 % alginate solution stored for 10 days at 23°C or 3 days at 4°C.* ■ *- freshly prepared solution;* ● *- 1 day, 23°C;* ▲ *- 1 day, 4°C;* ▼ *- 2 days, 23°C;* ◆ *- 2 days, 4°C;* + *- 3 days, 23°C;* × *- 3 days, 4°C;* ✳ *- 7 days, 23°C;* _ *- 8 days, 23°C;* | *-10 days, 23°C. Curves are fitted using equation 5.3 ($\mu_a = K\dot{\gamma}^{n-1}$).*

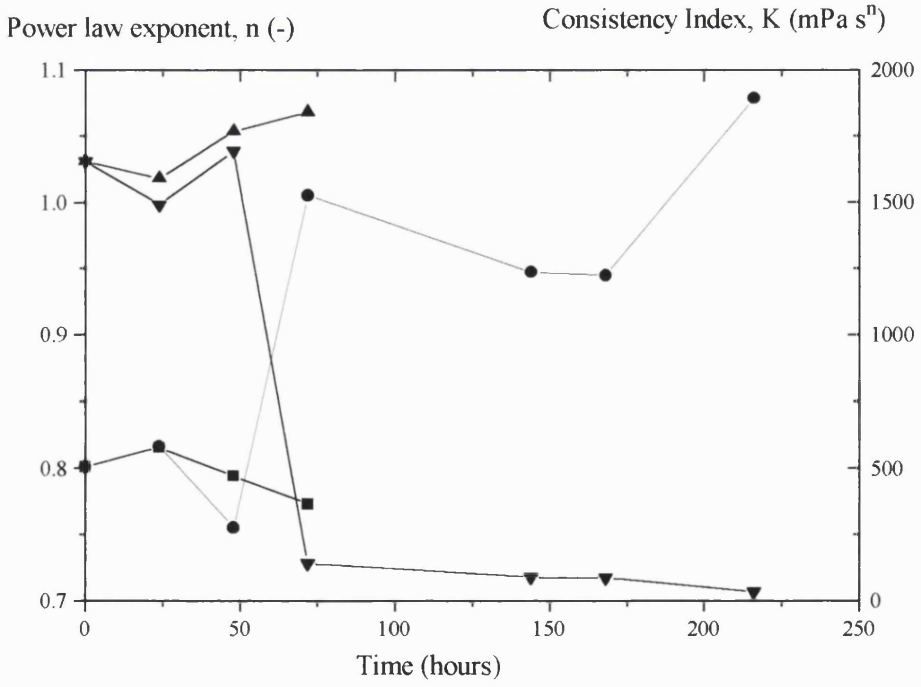


Figure 6.10. *Values of K (mPa s^n) and n (-) for 140 mg/mL protein / 1.3 % alginate spinning dope versus storage time.*
■ *- n, 4°C;* ● *- n, 23°C;* ▲ *- K, 4°C;* ▼ *- K, 23°C.*

Figures 6.9 and 10 show data for the storage of 140 mg/mL protein / 1.3 % alginate spinning dopes at either 23°C over a period of 10 days or 4°C over 3 days. Solutions stored at either 23°C for 1 day or 4°C for 2 days had the same apparent viscosity as a freshly prepared solution. Apparent viscosities at shear rates of $1000s^{-1}$ are used for comparison. Storage for 3 days at 4°C resulted in a 7 % decrease in viscosity whereas a solution stored at 23°C for 2 days showed a 25 % decrease from its initial viscosity. Storage for 3 days and 10 days at 23°C resulted in only 31 and 12 % of the original viscosity being maintained respectively. A decrease in viscosity with time corresponds to the loss of the solution's spinnability. The consistency indices, K, for solutions stored at 23°C initially and after 3 and 10 days were 1600, 150 and 31mPa s^n respectively, confirming a decrease in apparent viscosity. The power law exponent increased from 0.8 to 1.0 between days 2 and 3, representing the loss of shear thinning behaviour. Solution spinnability was also lost. Values for K and n and solution spinnability were maintained for dopes stored for 3 days at 4°C.

From this work it was seen that overnight storage of either dope at 23°C does not adversely affect the apparent viscosity of the spinning dope. However, if the spinning dope was to be made in a continuous process, following on from fibronectin extraction from plasma and then stored ready for use, a different formulation would be required. The high protein dopes showed a much greater viscosity loss than the high alginate dopes suggesting that the drop in viscosity and loss of spinnability is due to protein breakdown. This may be due to enzymic degradation or bacterial digestion of the protein chains. The loss of shear thinning behaviour with time also suggests the breakdown of alginate in the solution.

Initially it was felt the use of 6 M urea as a solvent may also add a validatable viral inactivation step to the process. The need to increase the protein concentration for pilot scale spinning led to dilution of the urea concentration from 6 M to 2 M by buffer associated with the protein precipitate. To avoid this problem solid urea was added to the precipitate with a small volume of phosphate buffer so that the final urea concentration in the system would be 6 M. The effect of storage for up to 3 weeks was examined for solutions prepared in the original way and with the addition of solid urea. Results are shown in Figures 6.11-14.

Although alginate has been described with a number of wound dressing and tissue engineering applications, (see section 5.2.3.1) it was decided that the fibres made from the high protein dope would favour cell adhesion and so only these solutions were used for the following experiments.

Measurement of viscosity for the precipitate mixed with solid urea was carried out using the MS-108 cup and bob attachment rather than the MS-114 attachment previously used despite the MS-108 being more suitable for higher viscosity fluids. This was because the MS-108 requires only 0.75 mL of sample compared to 3.5 mL for the MS-114. 100 % glycerol was used to compare the accuracy of measurement of the two devices because glycerol has a similar viscosity to the fibronectin solution. For shear rates between 200 and $1000s^{-1}$ the MS-108 was found to measure the viscosity of 100 % glycerol as half that measured by the MS-114 (332 mPa s compared to 630 mPa s). Values for the fibronectin-fibrinogen solutions have been corrected accordingly (see Appendix 2, 10.2.3).

It was expected that when the protein precipitate was dissolved directly into the 6 M urea solution that the apparent viscosity would decrease within 2 - 3 days, Figure 6.9. The apparent viscosity at $1000s^{-1}$ decreased with storage at 23°C from 490 mPa s to 71, 52 and 45 mPa s respectively after 1, 2 and 3 weeks respectively. For storage at 4°C the apparent viscosity decreased to 277, 135 and 100 mPa s over the 3 week period (Figure 6.11). Values of n and K for the solution stored at 23°C increased and decreased respectively during the first week whereas values for n and K for the solution stored at 4°C changed more slowly over the 3 week period (Figure 6.12). Some shear thinning behaviour was still present after 3 weeks storage at 4°C. Thus storage at 4°C reduces the loss of viscosity and shear thinning characteristics which can occur during long term storage for the high protein solution.

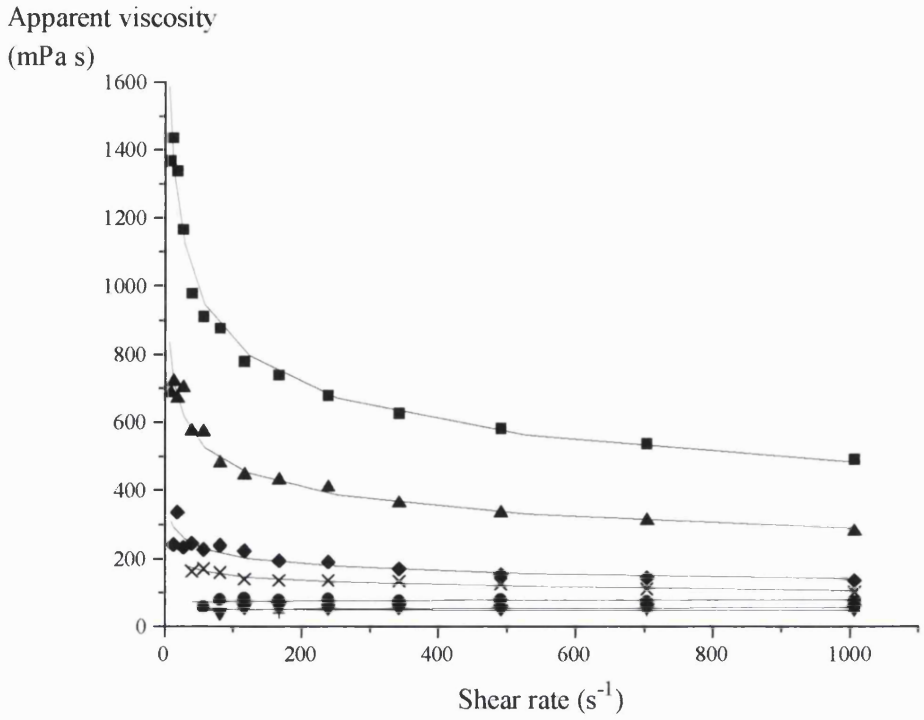


Figure 6.11. *Shear rate vs apparent viscosity for 140 mg/mL protein / 1.3 % alginate solution, stored for 3 weeks at either 23°C or 4°C. ■ - 24 hours after manufacture; ● - week 1, 23°C; ▲ - week 1, 4°C; ▼ - week 2, 23°C; ◆ - week 2, 4°C; + - week 3, 23°C; × - week 3, 4°C. Curves are fitted using equation 5.3 ($\mu_a = K\gamma^{n-1}$).*

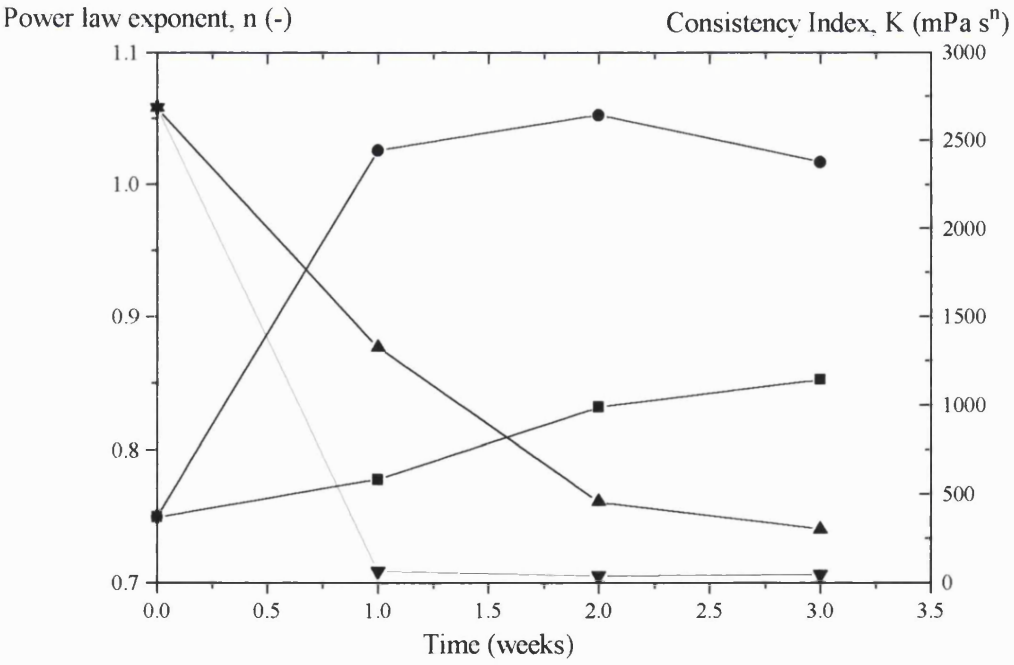


Figure 6.12. *Values of K (mPa s^n) and n (-) for 140 mg/mL protein / 1.3 % alginate dissolved in 6 M urea solution. Storage was at either 23°C or 4°C over 3 weeks. K and n values calculated for range of shear rates 7 - 1000s^{-1}.■ - n, 4°C; ● - n, 23°C; ▲ - K, 4°C; ▼ - K, 23°C.*

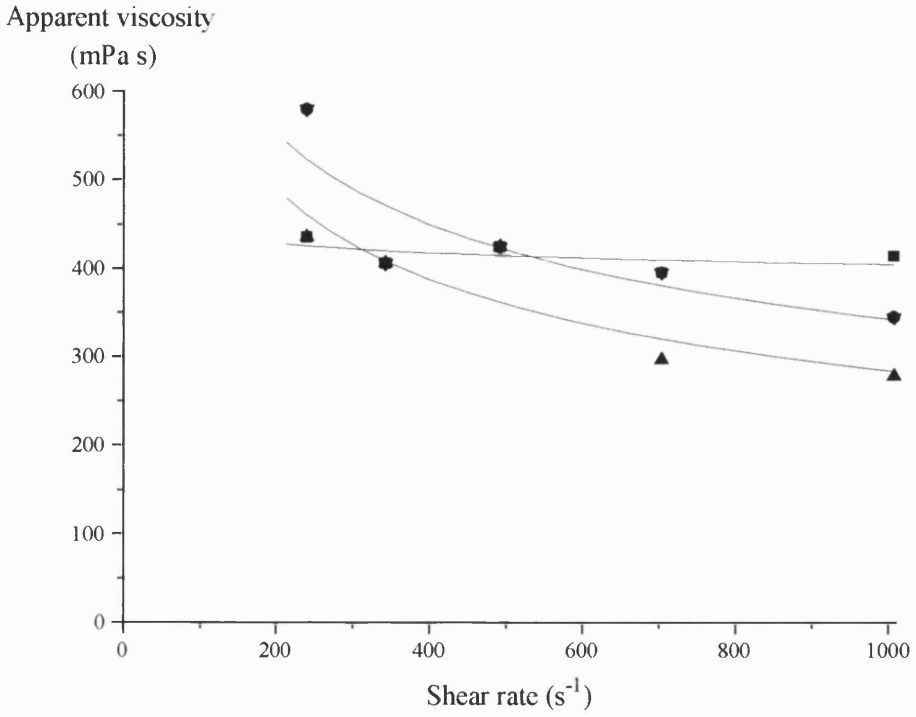


Figure 6.13. *Shear rate vs apparent viscosity for 140 mg/mL protein / 1.3 % alginate with addition of solid urea (6 M). Values were measured using the MS-108 cup and bob device. Solutions were stored at 4°C but viscosity measurements were made at 23°C.* ■ *- start;* ● *- week 1;* ▲ *- week 2;,* ▼ *- week 3. Values calculated for shear rates 170-1000s^{-1}. Curves are fitted using equation 5.3 ($\mu_a = K\gamma^{n-1}$). Curves for weeks 1 and 3 are identical.*

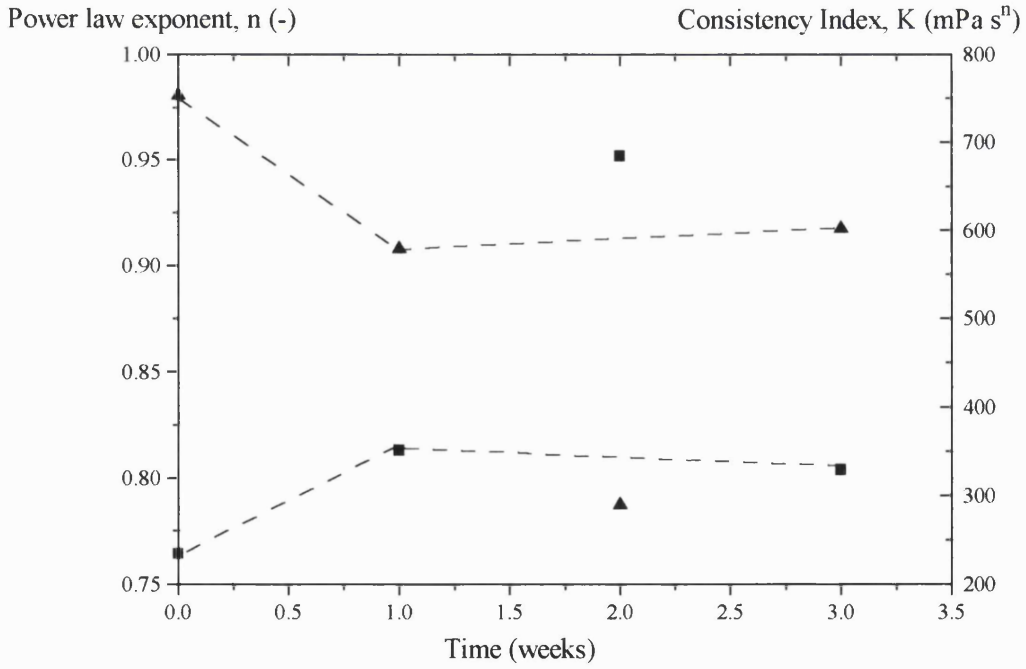


Figure 6.14. *Change in K (mPa s^n) and n (-) values for spinning dope consisting of 140 mg/mL protein / 1.3 % alginate with the addition of solid urea up to a concentration of 6 M.* ● *- n;* ▼ *- K. Solutions were stored at 4°C but viscosity measurements were made at 23°C after the solutions had reached room temperature.*

When the solid urea was added to the protein precipitate and the phosphate buffer, the resulting solution was clear and colourless. Previously the spinning dopes had retained small amounts of dispersed precipitate giving them an opaque appearance.

The apparent viscosity of the solution, containing solid urea, shortly after it was prepared was 425 mPa s at $1000s^{-1}$ and the solution had little shear thinning character. Measurements made after 1 and 3 weeks had apparent viscosities of 340 mPa s at an equivalent shear rate (Figure 6.13). Values were lower after 2 weeks, 275 mPa s, although this was attributed to experimental error. K and n values also remained relatively constant over the three week period, Figure 6.14. Shear thinning behaviour was still present at week 3 although addition of solid urea reduced the shear thinning properties of alginate. The initial value of n where solid urea has been added was 0.98 describing Newtonian behaviour. In contrast, protein precipitate that had been dissolved in 6 M urea solution gave n = 0.74.

Addition of solid urea resulted in the apparent viscosity of the solution after 3 weeks storage at 4°C being reduced to 83 % of the original value. Fibres could still be wet spun from this solution. Where the precipitate had been dissolved in a solution of 6 M urea the equivalent apparent viscosity was 20 % of its original value and fibres could not be spun from the solution. From this it can be seen that maintaining the concentration of urea at 6 M improves the prospects for long-term storage of the spinning solution. The high urea concentration probably reduces bacterial breakdown of both alginate and protein. The use of precipitate prepared under the clean conditions would be necessary for a Good Manufacturing Practice regulated process (chapter 8) and would reduce microbial contamination and prevent the breakdown of protein with time. Protein breakdown may also occur due to traces of proteolytic enzymes in the fibronectin - enriched cryoprecipitate. If this were the case then enzyme inhibitors may have to be added to the solutions to prevent a viscosity reduction. This would also reduce the rate of breakdown of the final fibre.

6.5. Summary

The aim of this work was to assess the feasibility of wet-spinning fibronectin / fibrinogen fibres on a pilot scale spinning rig with a view to reliable and reproducible production of fibres.

Dope preparation and set-up of spinning rig

Dopes equivalent to those used for small scale spinning of fibronectin / fibrinogen protein fibres could not be used successfully on the pilot scale rig. The solution viscosity was increased by dissolving more protein precipitate into a 6 M urea solution to bring the protein concentration to 140 mg/mL but a side effect of this was the dilution of the urea concentration to 2 M. Viscosity could also be increased by increasing the alginate concentration.

Extrusion of these solutions through a spinneret, diameter 1 mm, length 18 mm, was found to produce the best fibres when the protein was coagulated in 0.25 M HCl, 2 % $CaCl_2$, pH 0.9.

Fibre properties

Fibre production was continuous and thus more reproducible than the small scale spinning method. Fibre properties differed depending on their treatment and composition. Acetone could be used to dehydrate the fibres and improve their tensile properties. Although not as strong as the twisted, drawn cables, the fibres possessed superior flexible properties, which aided processing. The fibres also possessed ultrastructural orientation in the form of ridges on the fibre surface, which may serve to orientate cells in the same manner as the drawn fibronectin cables.

Storage of dopes

A decrease in viscosity with long-term storage of the dope prevents the design of a continuous process from cryoprecipitate to spinning dope. Preliminary results suggest maintaining a urea concentration of 6 M and storage at 4°C may help reduce the protein /alginate degradation.

7.0. Fibronectin cables attached to coliagen sponges - preiiminary results of a test for orientated skin replacement.

7.1. Introduction

Human dermal fibroblasts have been shown to orientate themselves both on top of fibronectin cables and alongside, for distances up to 900 μm. The distance over which cells formed aligned bands was found to be independent of cable width (Harding, 1996; Harding *et al.*, 1998). This chapter describes the use of these cables as a preliminary scaffold for the preparation of orientated sheets of human dermal fibroblasts. Cell orientation was deemed important because cells orientated in one direction are believed to produce new extracellular matrix which is orientated in the same direction as the cell. Collagen fibrils of the extracellular matrix of non-injured dermis are arranged as an interlocking network and so for use as a dermal replacement it was envisaged that a number of sheets of orientated cells could be placed into a deep wound as layers with each layer having cells orientated at a different angle. The final result, it is hoped, would be a network of collagen fibrils resembling that in natural tissue and generated by controlled cell orientation. This control of cell orientation should prevent the random generation of extracellular matrix, which can lead to scarring.

To observe the orientation of cells on a single sheet using a minimum amount of fibronectin, and thus reduce the volume of plasma required, fibronectin cables were spaced out at distances at least 1 mm apart on a collagen backing material, as described in section 2.5.2. These results form a preliminary investigation to determine whether human dermal fibroblasts would form an aligned sheet of cells using directional cues supplied by fibronectin cables and collagen sponge as a backing layer. Keratinocytes were not included in this investigation.

Collagen sponges were chosen as a backing material for the fibronectin cables because they have been used extensively to mimic the dermis in the development of skin substitutes (Yannas *et al.*, 1980; Bell *et al*, 1981; Suzuki *et al.*, 1990; Matsuda *et al.*, 1993; Doillon *et al.*, 1994; Maruguchi *et al.*, 1994; Yannas, 1995) and have shown little

antigenicity when injected into volunteers (Wilkins *et al.*, 1994). However bovine collagen is frequently used to prepare such sponges, posing a potential regulatory problem due to possible prion contamination (chapter 8.0).

7.2. Dermal fibroblasts on collagen sponges

The collagen sponges used were fibrillar with channels running into the centre of the sponge. Pore size was measured with respect to area and a wide range of pore sizes was seen, < 150 - 23 100 square μm. Linear dimensions for these pores were < 12 x 12 μm to 113 x 203 μm. Some pores were too large to be viewed on a single micrograph. A scanning electron micrograph of a sponge with fibronectin cables but no cells is shown in Figure 7.1a.

After 1 day in culture, cells could be seen attached to the surface of the cable but were difficult to distinguish on the surface of the collagen sponge. Further examination after 3 and 7 days revealed cells both confluent and aligned on the fibronectin cable. Fourteen days after the start of culture, cells were visible both on the collagen sponge and on the fibronectin cable. Figures 7.1b and c show sponges after fourteen days in culture with no cells and seeded with fibroblasts respectively. Cells were visible on the seeded sponge giving a much denser appearance to the sponge surface. Examination of the fourteen day, cultured sponge at higher magnifications (Figure 7.1d) showed regions of cellular alignment on top of the cable and on the sponge to the left of the cable. This second band of aligned cells to the left was unlikely to be due to guidance cues from the fibronectin cable because there was no continuous band of cell alignment between the cable and the narrow band of alignment. Instead this may have been due to very fine fibronectin strands which were added to the sponge during manufacture or due to the arrangement of the collagen fibrils. Alignment of fibroblasts adjacent to the cable with the collagen sponge backing was not as great as the 900 μm wide bands seen with glass coverslips as a backing sheet (Harding, 1996; Harding *et al.*, 1998). This is probably due to the more random directional cues provided by the fibrillar collagen sponge.

Ascorbic acid was added to the medium because it has been shown to be essential for collagen synthesis and the assembly of cross-linked collagen fibrils into bundles and thus

the formation of the new extracellular matrix (Grinnell *et al.*, 1989). Figure 7.1e is a high magnification scanning electron micrograph of cells aligned on the fibronectin cable after 14 days. Harding (1996) noted fine collagen fibrils produced by fibroblasts when cultured on fibronectin cables. Such fibrils are also seen in this micrograph and are broadly orientated parallel to the cell's longitudinal axis. Unfortunately the type of collagen and its age cannot be estimated from these micrographs.

The range of pore sizes was estimated by measurements taken from scanning electron micrographs of the original sponge with no cells, (Figure 7.1a) sponge with cells and fibronectin after 1 (not shown) and 14 days in culture (Figure 7.1c) and sponge with no cells after 14 days (Figure 7.1b). Results are shown in Figure 7.2. and Table 7.1 explains the classification of pore sizes. In all cases the majority of pores are small, less than 149 square microns. There was no significant difference between the frequency of pore sizes measured and sample treatment except for the uncultured sponge and the sponge with no cells after 14 days in culture medium ($p=0.013$, Independent t-test, 95% confidence level) for the smallest pore size range. This was an unexpected difference and may be ruled out with a larger sample size. It was expected that the cells would form a continuous sheet across the sponge, reducing the pore size. However the pore size measurements demonstrate that this is not the case and it appears that the cells follow the collagen fibrils and lie around the edges of the pores, as seen in Figure 7.1c. Since some of the pores are relatively large and form channels into the sponge, it would appear that cells also migrate into the centre of the sponge. Cross-sections of the sponge could be taken and stained to confirm this. Cells would ideally be situated both in and on top of the collagen sponge for use as a skin replacement. Cells trapped inside the sponge would produce new matrix and aid the replacement of dermal tissue although it is unlikely that the directional cues of the fibronectin cable would extend into the centre of the sponge.

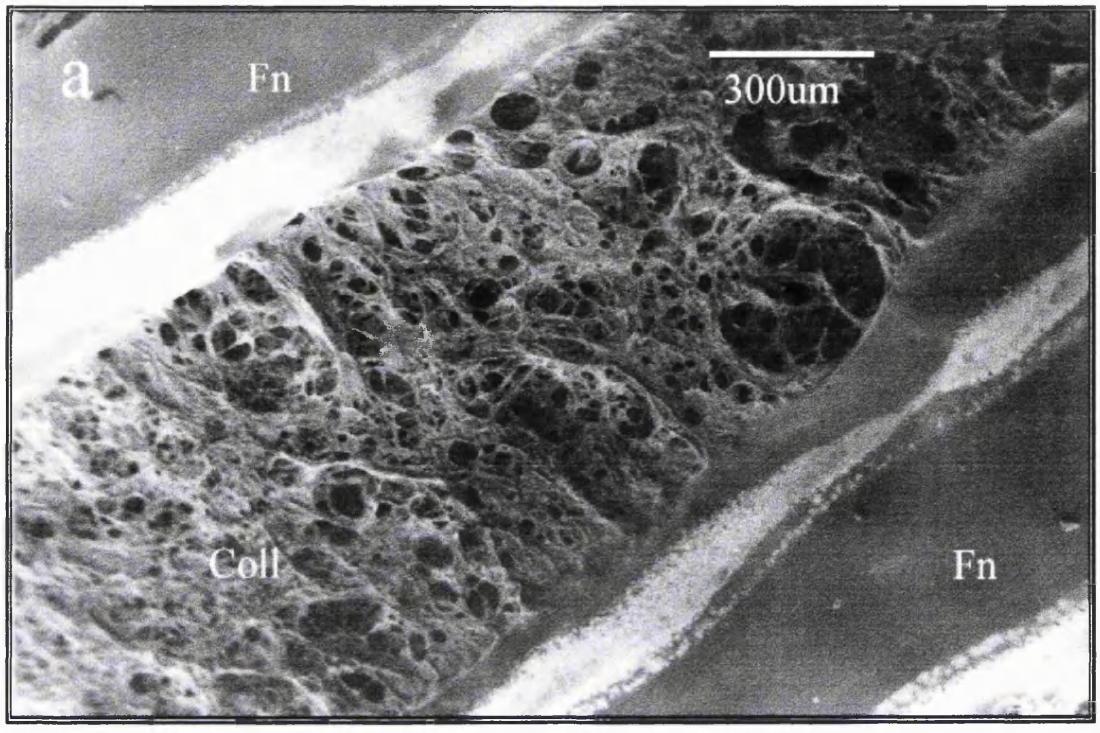


Figure 7.1a. *Collagen sponge (Coll) with two fibronectin cables (fn) attached. Cables were drawn on to the top of the sponge, the entire sponge washed to remove acid remaining from the cable manufacturing process and then freeze dried overnight.*

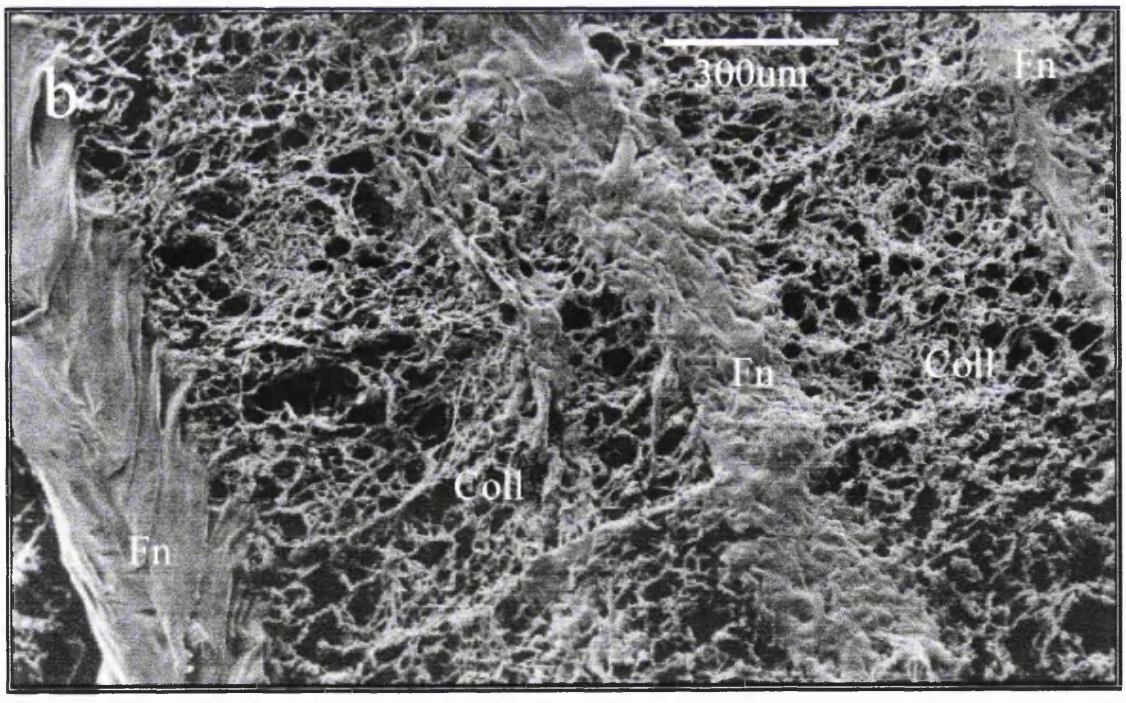


Figure 7.1b. *Collagen sponge (Coll) with fibronectin cables (fn) which has been incubated in DMEM at 37°C for 14 days with no cells present. The cables have remained firmly attached to the sponge and the sponge has not swollen to occlude the pores.*

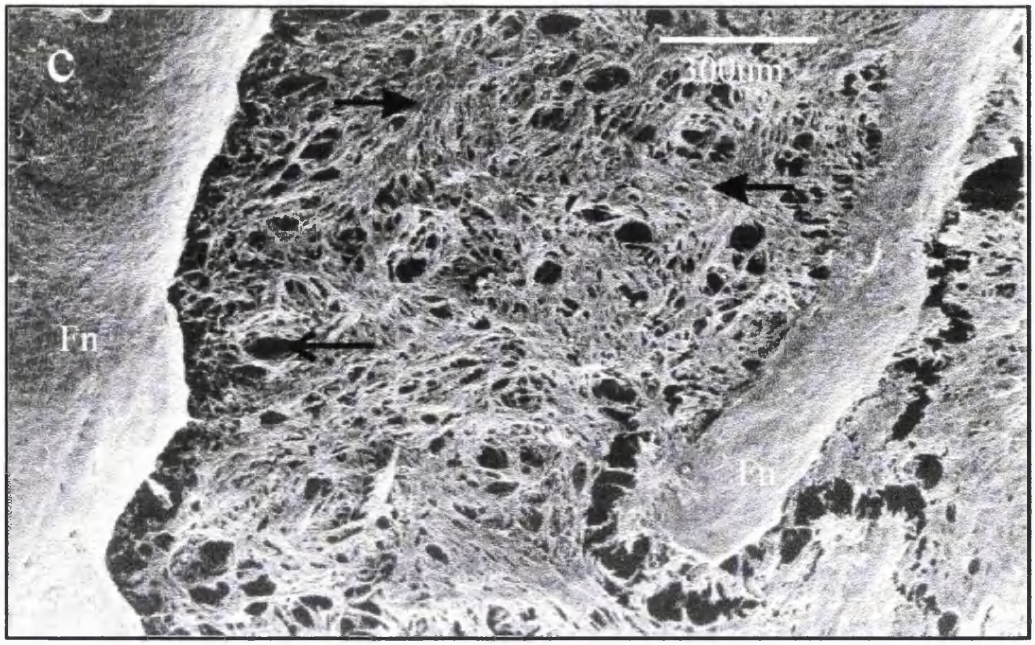


Figure 7.1c. *Collagen sponge with fibronectin cables (fn) seeded with human dermal fibroblasts after 14 days in culture. Solid arrowheads indicate regions of the sponge densely populated by dermal fibroblasts. These cells appear to use the fibrillar network of the sponge to guide their orientation and avoid the larger pores (open arrowhead). Fn cables are covered with a confluent layer of cells.*

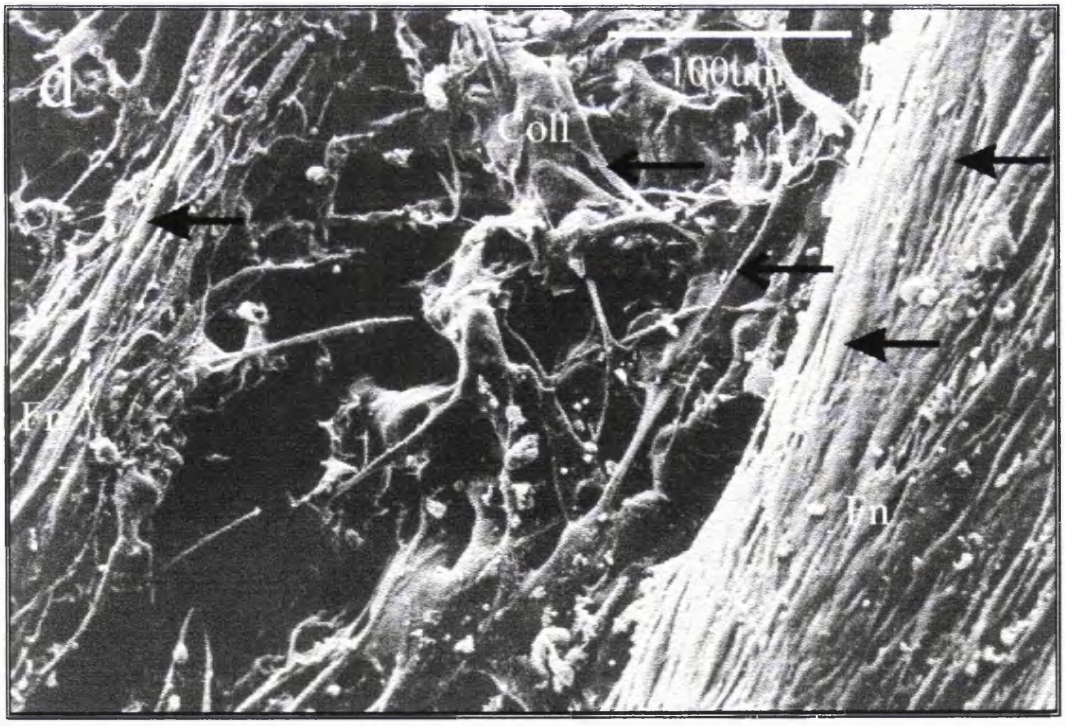


Figure 7.1d. *Higher magnification view of sample in Fig 7.1c. showing dermal fibroblasts aligned (solid arrowheads) on the main fibronectin cable to the right of the micrograph and the narrower region of fibronectin to the left. Fibroblasts are also visible on the collagen sponge (open arrowheads) although alignment of these cells is again governed by the fibrils of the sponge.*

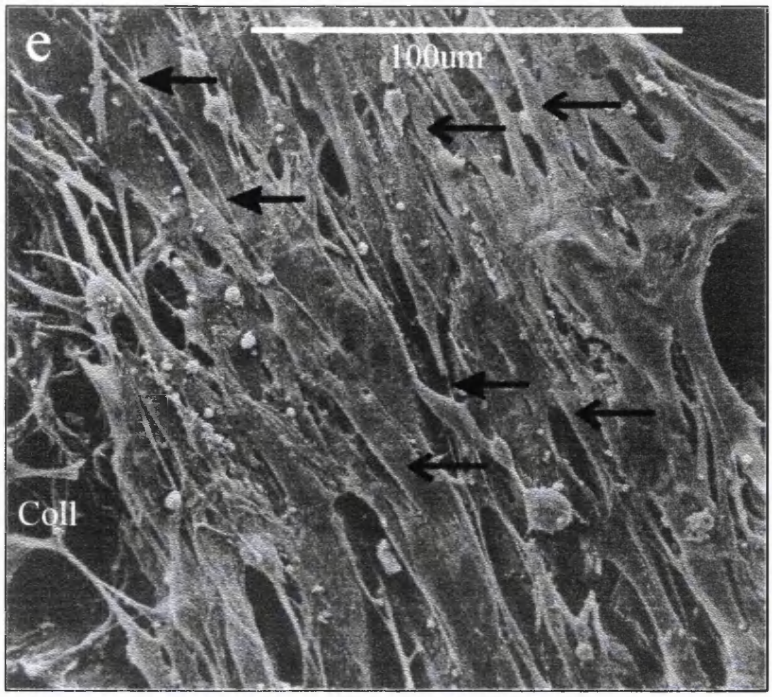


Figure 7.1e. *High magnification scanning electron micrograph showing dermal fibroblasts aligned on a fibronectin cable. The collagen sponge is visible to the left of the micrograph and cells are attached to both the collagen and the fibronectin. A number of cell bodies are indicated with open arrowheads and are aligned in one direction, parallel to the longitudinal axis of the fibronectin cable. This supports well work by Harding (1996). The morphology of the cells is the typical elongate, bipolar one of anchored, dermal fibroblasts, 10 μm wide and up to 100 μm in length, with extending processes. Fine fibrils are also visible, solid arrowheads, and could be newly formed collagen fibrils forming part of the new extracellular matrix or they may be fine cell processes. Specific markers are required to confirm the identity of these fibrils.*

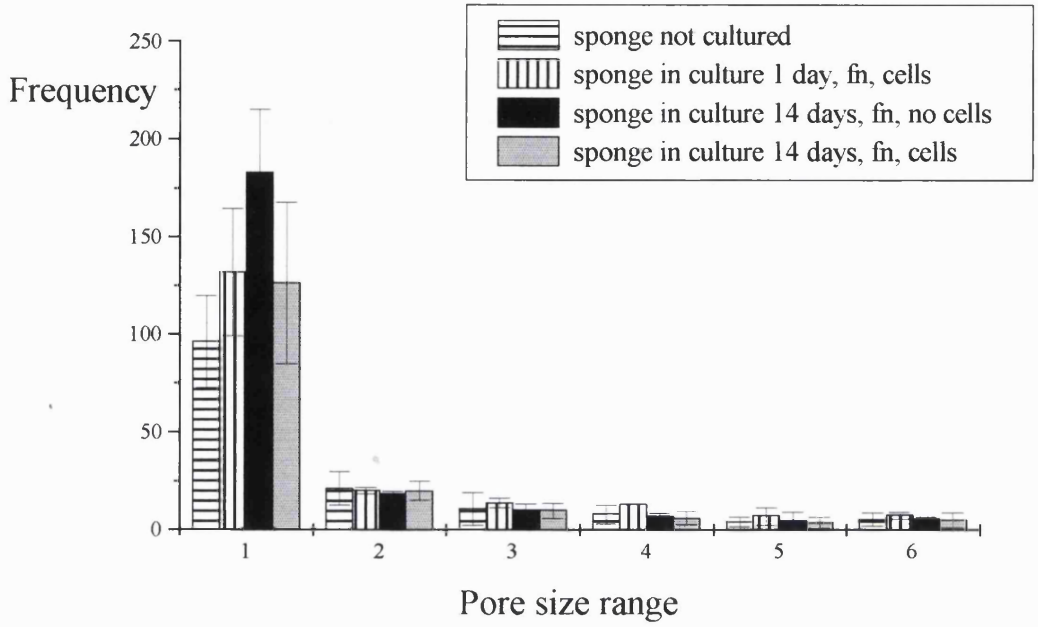


Figure 7.2. *Frequency of pore size measurements for collagen sponge samples. All samples were prepared as described in section 2.5.2. Pore size measurements were made from scanning electron micrographs. Three micrographs were taken per sample and the size of all pores contained within five 2x2 cm squares for each micrograph were measured. Results are expressed as an average number of pores seen in the tested areas per micrograph ± 95% confidence interval. Details of pore size range are given in Table 7.1.*

Pore size range	Area measured on micrograph (square mm)	Measurements with error accounted for (square mm)	Actual sponge pore area (square μm)
1	≤1	≤2.25	≤149
2	>1, ≤4	>2.25, ≤6.25	>149, ≤413
3	>4, ≤9	>6.25, ≤12.25	>413, ≤810
4	>9, ≤16	>12.25, ≤20.25	>810, ≤1338
5	>16, ≤25	>20.25, ≤30.25	>1338, ≤2000
6	>25	>30.25	>2000

Table 7.1. *Classification of pore size ranges for Figure 7.1. The dimensions of the pores were measured in mm from the scanning electron micrograph. Assuming that the pores were approximately square or rectangular, these dimensions were then converted to an area. As measurements were only made to the nearest millimetre, a certain amount of error in the classification was seen. To account for this the range of areas represented by the groups was adjusted (column 2) to include pores with dimensions 0.5mm greater than those measured. These measurements were calibrated from the SEM magnification to give a pore area range in square μm*

For use as a skin replacement therapy the collagen backing material would have to remain in the body for a period of weeks to months for cells to produce orientated matrix and the wound to heal. The sponges used had been treated, during manufacture, with diphenylphosphorylase (DPPA), a cross-linking enzyme which has been shown to decrease the rate of sponge biodegradation (Rault et al., 1996) whilst demonstrating better cytocompatability than other cross-linking agents, such as glutaraldehyde (Petit *et al.*, 1994). DPPA acts by forming an acyl azide group on the carboxyl groups of aspartic and glutamic acid residues in collagen. This azide groups then bind to lysine and hydroxylysine residues on other collagen fibrils (Petit *et al.*, 1994). Long term culture experiments are required to demonstrate the sponge stability in culture.

A number of groups have investigated seeding fibroblasts onto collagen sponges as a method of producing a tissue repair scaffold. Berthod *et al.* (1993) used a collagen sponge, containing chondrotin 4-6 sulphate and chitosan, in preference to a collagen gel because the sponge porosity (size range 50-150 μm) avoided cell confinement and allowed the production of extracellular matrix and reduced matrix contraction. Cells which are closely packed into a matrix are believed not to produce new material due to a feedback inhibition method. Further work by the same group, demonstrated that after 10 days human fibroblasts were seen to migrate into the sponge and show a high level of collagen and protein synthesis. New collagen production was highest on days 1-3 and there was a 3 fold increase in the number of cells between days 3 and 11. After day 11, the cell number reached a plateau and between days 10 and 27 the newly formed matrix had filled the pores of the sponge and the biosynthetic level had decreased (Berthod *et al.*, 1996). After 14 days the cells used in the fibronectin cable/ collagen sponge experiments had nearly formed a confluent layer and new matrix was visible (Figures 7.1c, e). However the results were difficult to quantify from the electron micrographs and methods of estimating cell number and collagen production should be used to produce quantitative data. Prolonged culture should also demonstrate any further effects on cell orientation.

Collagen sponges with a variety of glycosaminoglycans have been tested for use as a cell scaffolds (Yannas, 1995). The addition of hyaluronate and fibronectin to a collagen sponge has been shown to increase new, cell induced collagen synthesis and deposition of the protein around the cells, as well as increasing cell growth rate, cell division and migration into the interior of the sponge. Fibronectin was found to act as a chemoattractant and to increase fibroblast spreading when sponges with a pore size range of 94-214 μm were used (Doillon and Silver, 1986; Doillon *et al.*, 1987). Based on the work by Doillon *et al.*, even if perfect alignment is not achieved on top of the collagen sponge the use of fibronectin as an adjunct would aid cell attachment and migration. The collagen sponge could also act as a depot for growth factors, in a similar manner as that described for the original fibronectin mats (Brown *et al.*, 1994). To achieve improved alignment, less fibrillar backing materials may be required. Possibilities include agar, hyaluronate, collagen or fibrin films. It is important when choosing a backing material that the new 'tissue' will not be implanted in a large, solid volume. Cells will not survive if they are > 100 μm from the nearest functional capillary (Langer et al, 1995) and the new tissue must also be porous enough to allow vascularisation to occur. Hubbell and Langer (1995) describe the most successful scaffold made during the development of Dermagraft, as 90 μm thick, with pores of diameter 200-220 μm. If smaller pores were used, then cells did not enter the centre of the sponge but if they were much larger, the cells could not bridge the gaps between pores and the production of proteins and proteoglycans was reduced.

7.2.1. Freeze drying

Yannas (1995) described freeze drying as a method of altering collagen sponge pore size. Fast freezing produces smaller ice crystals in the protein and thus smaller pores. Tests with freezing 140 mg/mL protein / 1 % alginate fibres at -80, and -20°C and also air drying, before freeze drying for 24 hours did not appear to affect the surface of the fibres, as examined by scanning electron microscopy. However, examination of the interior of the fibres and further focus on the drying technique to alter material porosity may be critical for use of the fibres where cells are required to migrate through the centre of the fibre as well as on top of it, e.g.: in nerve repair conduits.

7.3. Summary

The aim of this work was to form a sheet of aligned human dermal fibroblasts on a collagen backing sheet using fibronectin cables to provide the directional cues.

Human dermal fibroblasts attached to both the fibronectin cable and the collagen backing material and produced a virtually confluent layer after 14 days in culture. Cell orientation on the cable was excellent but more random on the sponge due to directional cues provided by the sponge itself. To produce an orientated sheet of cells, a more homogeneous backing sheet may be required. However some orientated new collagen fibrils were seen and the directional scaffolding provided by the fibronectin cables may be enough to improve the quality of wound repair, whilst the collagen sponge acts as a dermal equivalent. The collagen sponge also acts as a medium for cell transplantation. More quantitative data is required, particularly for measuring the number of cells in the sponge and for new collagen production. Longer term experiments should also be carried out to examine sponge and fibronectin stability and matrix production as well as the effect of fibronectin cables on keratinocyte orientation.

8.0. Process design and Good Manufacturing Practice issues

8.1. Introduction

All products intended for human medicinal use must be manufactured according to guidelines entitled "current Good Manufacturing Practice (cGMP)" to ensure that they are effective, safe and consistently manufactured and controlled to the quality standards appropriate to their intended use.

Before medicinal products enter the marketplace they must first pass through a review and approval stage. The method of review of tissue engineered products made in the US appears to depend on the FDA's classification of the product type. Apligraf®, a skin replacement product, manufactured by Organogenesis, containing both human keratinocytes and fibroblasts but no synthetic component was reviewed as a medical device and has now been approved by the FDA for treatment of venous leg ulcers. However Genzyme Tissue Repair's Carticel, an autologous cartilage cell transplantation therapy, was first considered as a medical device and then as a biologic by various regulatory centres in the US and was initially unregulated. Under a new set of regulations released by the FDA, Carticel became regulated because it contained autologous cells (Brower, 1997). The premarket approval for Advanced Tissue Sciences' burn treatment product, Dermagraft TC™, was given an expedited review by the FDA and clinical trials were conducted under a Investigational Device Exemption (Johnson Langer, 1996). This product is now on the market. A related product, Dermagraft, containing metabolically active cells and with prospective use for the treatment of diabetic foot ulcers was approved by the FDA's General and Plastic Surgery Devices Advisory Committee on the condition that a post-marketing study was carried out to provide additional efficacy data (Seachrist, 1998). However more recently the FDA have asked Advanced Tissue Sciences to resubmit their premarket approval for Dermagraft after new trial data has been collected. Both Dermagraft TC™ and Dermagraft are based on scaffolds constructed from suture materials, previously approved by the FDA for human use (Hubbell and Langer, 1995).

This chapter describes how these guidelines, set by the appropriate regulatory bodies, should be applied to the production of fibronectin fibres.

8.2. A suggested process flowsheet

From the work described in this thesis a suggested process flowsheet for the production of fibronectin/fibrinogen fibres has been developed, Figure 8.1. The manufacture of the material takes place in three main stages:

- the preparation of the fibronectin/fibrinogen feedstock
- wet spinning the fibres
- manipulating the fibres into the final product.

However each individual step must be optimised so that the final fibres have the necessary properties for their function. As previously described, the method of preparation of the fibronectin / fibrinogen feedstock can be altered to produce varying ratios of the two proteins, section 3.3, and this in turn may alter the cell adhesion properties of the material. The addition of alginate, as a viscosity enhancer, reduces the material's self-adhesive properties and may prevent attachment to a backing sheet whilst the type and strength of acid used in the coagulation bath will affect the rate of coagulation and therefore the structure and physical properties of the final fibre. Fibres can be drawn to different diameters - large diameter fibres may be used as single fibres on their own, whilst finer fibres can be attached to a backing sheet. The maximum diameter of the fibre which can be wet spun is limited by the diffusion of coagulant into the centre of the fibre.

Single fibres could be used as conduits for nerve or tendon repair, both applications requiring a high degree of cell orientation, whilst sheets of fibres on a backing material could be used to orientate cells over a large area and find use in skin repair and replacement. Fibres or sheets, formed by the described process, could be rehydrated and implanted into wounds or gaps. Cells in the wound margin, or severed stump of tendon or nerve, would then migrate onto the fibronectin and be supported and aligned in the main wound area or guided along the fibre to the other side of the gap. These cells.

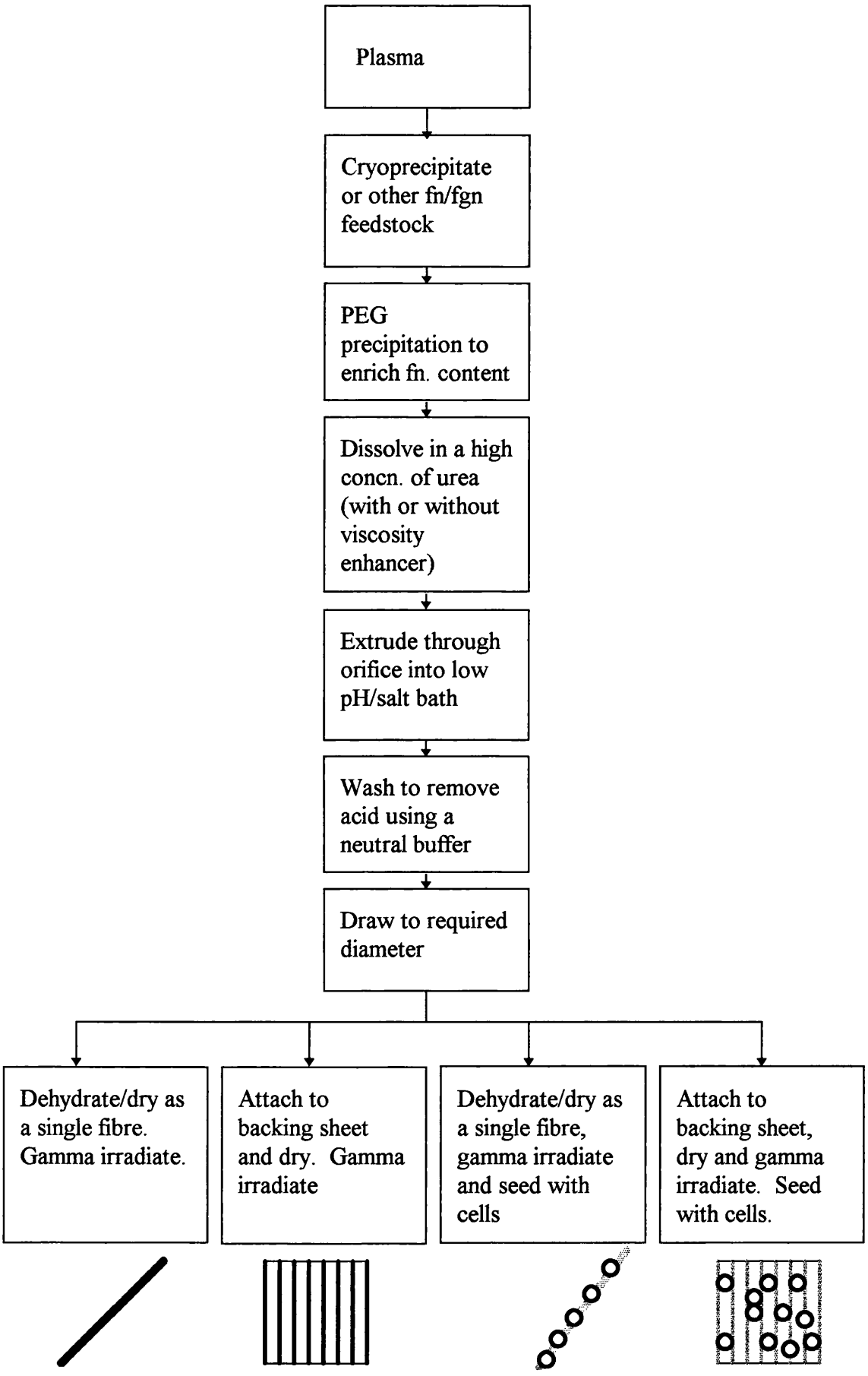


Figure 8.1. *Proposed flowsheet for manufacture of fibronectin/fibrinogen fibres from plasma.*

would then start to produce their own new extracellular matrix to heal the wound. This is probably acceptable for small area wounds or gaps. For larger wounds e.g.: non-healing ulcers and extensive burns, it may be beneficial to have dermal fibroblasts already established in the material and producing their own matrix before the material is implanted. Some wounds, such as non-healing ulcers, provide hostile environments for cells and thus a supply of new cells with the skin repair matrix would be beneficial. The possiblity remains to use these fibronectin materials as depots for growth factors (Brown *et al.*, 1994) which are absent or rapidly degraded in the wound environment.

In order to finalise the process, the final properties of the product required must be established, and the process optimised accordingly. The manufacture of any material for human (or veterinary use) is regulated so that infection and toxicity are avoided and reproducibility is maintained. Sections 8.3 and 8.4. discuss the current state of regulation for tissue engineered products and Good Manufacturing Practice (GMP) issues relevant to the process flowsheet.

8.3. A summary of current regulatory issues for tissue engineered products

The recent global increase in the production and diversity of tissue engineered products has resulted in a review of regulations set by both the FDA (Food and Drug Administration, US) and the European Commission concerning the way these products are regulated. Efforts are underway to produce guidelines which are consistent worldwide to develop a ' harmonized regulatory strategy' (Hellman, 1997). The regulation of tissue engineered products will be overseen by the national agency responsible for their approval and the focus will be set on product safety and efficacy, preclinical evaluation, clinical investigation, and postmarket requirements. Included in these issues are testing and process validation for adventitious agents, cell and tissue sourcing and characterization, characterization and demonstration of compatibility of biomaterial components, product consistency and stability, preclinical *in vitro* and animal models, clinical study design and endpoints for clinical safety and efficacy (Hellman, 1997).

A number of factors determine how a product will be regulated. In the US, tissue engineered products can be classified as a device, a biologic or a drug although products rarely fit easily into only one of these categories (Burlington, 1997). Examples of the three categories are given in Table 8.1.

Category	Example of product in category	Regulated in the US by:
Device	Medical devices and diagnostic kits (excluding biologics), cultured skin products, non-living human and animal tissues and implants	Centre for Devices and Radiological Health (CDRH)
Biologic	Intact microorganisms, cell culture derived protein or carbohydrate products, protein products produced by genetic alteration, vaccines, in vivo diagnostic and therapeutic allergenic products, blood-derived products, mono- and polyclonal antibodies, gene and cell therapies and biologic devices or diagnostic kits.	Centre for Biologics Evaluation and Research (CBER)
Drug	Naturally occurring chemicals, mineral, plant, animal or human tissue derived drugs, antibiotics, hormones, fungal and bacterial products and chemically synthesised molecules	Centre for Drug Evaluation and Research (CDER)

Table 8.1. *Summary of U.S. classification system and reviewing centres for new medical devices, biologicals and drugs* (Eggerman and Patterson, 1997).

The FDA has established an InterCentre Tissue Engineering Initiative to aid evaluation of tissue engineering applications and to identify areas for further study. This group has members from the three centres detailed in Table 8.1 as well as delegates from the Centre for Vetinary Medicine (CVM) (Johnson Langer, 1995). In the European Union, the Medical Devices Directive concerns implantable products containing non-viable animal tissue whilst control for human and all viable animal cell products is dealt with on a country by country basis (Durfor, 1997). The European Commission issued a Directive (93/42/EEC) relating to medical devices other than implantable medical devices and *in vitro* diagnostic medical devices. However this directive does not apply to human blood, blood products, plasma or cells of human origin. Amendments to this directive are under consideration to include tissues or substances of human origin which are non-viable or

rendered non-viable (Freeman, 1997). Freeman states that it is unlikely that viable tissues of any origin will be covered by the European Commission Devices Directives so these products will be examined according to national rules or may have to be covered by international initiatives.

In February 1997, the FDA released a set of proposed guidelines for the regulation of cellular and tissue-based products (FDA, 1997). These guidelines state that the use of autologous cells in tissue engineering will not require regulatory approval providing that they are removed and transplanted back into the patient in a single procedure. Tests for infective agents are recommended for autologous cells which are to be banked or processed and this is also a requirement for cells which will be used for allogeneic transplantation.

These new FDA guidelines also do not include blood products, which are already regulated elsewhere, although future developments may see a change in the regulation of traditional blood products towards those for cellular and tissue-based products. Animal products such as bovine collagen are not covered by these guidelines (FDA, 1997). The FDA state that combination products i.e.: combinations of cells and tissues with mechanical or synthetic components will be subject to premarketing requirements and regulated by the most appropriate centre (Table 8.1).

Classification of the fibronectin fibres would depend on their final form. Options for a final product described in section 8.2 included fibres alone, fibres with cultured cells, fibres attached to a backing material and fibres attached to a backing material plus cultured cells. From Table 8.1, the fibronectin fibres could be classified as either a device (cultured skin product or non-living human tissue) or a biologic (blood-derived product) and it would also depend whether cells are cultured with the fibre or not. The origin of the backing material e.g.: bovine collagen will also affect the final product's regulatory status.

The manufacture of products from fibronectin / fibrinogen may be covered by regulations for the production of plasma products rather than specifically for tissue engineering. However products containing cells and / or backing materials face a more complex regulatory procedure. The use of autologous cells would be preferred due to the

reduced risk of pathogen transmission and no immune response between host and donor cells. Cells would have to be removed from the patient and grown up to a high concentration either on the fibre / sheet or before seeding onto the material, before being returned to the patient. Even this technique, because it involves more than one surgical procedure and cell banking, is subject to scrutiny concerning possible virus transmission and bacterial contamination. According to these current regulations the FDA would regard any fibronectin scaffolds with cells as a combination product and review that accordingly.

The focus on the methods of tissue engineered product manufacture was on Good Manufacturing Practice regulation, which is applied to both pharmaceutical manufacture and blood products. These GMP issues are discussed in section 8.4.

8.4. Process GMP

Good Manufacturing Practice concerns the application of quality assurance to ensure that medicinal products are consistently manufactured and controlled to the quality standards appropriate to their intended use (MCA, 1997). A requirement of material production for clinical trials is manufacture in compliance with GMP. This section discusses how the Medicines Control Agency's rules and guidelines for pharmaceutical manufacturers and distributors can be applied to the process for spinning fibronectin / fibrinogen fibres.

8.4.1. General

Elements of GMP which are appropriate to the entire process will be discussed here whilst elements which are specific to a certain part of the process will be discussed in the relevant sections, 8.4.2 - 8.4.6.

8.4.1.2. Personnel

All personnel must be trained in all the procedures they carry out. They should also be trained in health and hygiene and the training must be kept up to date. Most manufacture will take place under clean conditions, so staff will be required to be appropriately clothed, with protective overalls, goggles, hair coverings, overshoes and gloves. There should be a member of staff responsible for each of the major stages of manufacture, including preparation of the feedstock, wet spinning and production of the final product and a 'Qualified Person' to ensure that manufacture of the fibronectin fibres is in accordance with directives and manufacturing authorities. All personnel would be required to pass both a laboratory and physical examination, including annual testing for markers for viral diseases (Kerner, 1995).

8.4.1.2. Premises and equipment

The premises used must be designed to avoid cross-contamination of the product. Thus the packaging and sterilisation of the final product must be carried out in a geographically distinct region to the wet spinning process. Wet spinning on a reasonable scale could be carried out in a laminar flow cabinet to ensure clean conditions. The flow of material to final product should pass through areas of increasing cleanliness and all areas should be easy to clean and maintain, with the appropriate temperature, humidity and air supply for the function. For example the area where the fibres are drawn to the required diameter may have to be closely temperature and humidity controlled.

Premises should be designed so that air flow through the building does not allow material contamination. For the aseptic manufacture of pharmaceuticals, positive pressures of 45 Pa have been used for the final filling stages, whilst post-virus and pre-virus inactivation procedures are carried out at 30 and 15 Pa respectively and the rest of the building is at 0 Pa. This prevents the flow of potentially contaminated aerosols to virus free areas. Equipment must not be swapped between such areas without sterilsation treatments (Kerner, 1995).

There should be standard operating procedures for all the equipment used, e.g.: centrifuges in feedstock preparation, the wet spinning rig, rollers to draw down the fibre, drying and sterilising apparatus. All equipment should have validated cleaning

procedures to prevent cross-batch contamination, e.g.: spinning batches of fibronectin with and without additives.

8.4.1.3. Documentation

The fibres must have a written specification to which each batch must conform. This will specify such things as proportion of each protein, % of additives, moisture content, presence and type of cells, fibre diameter, type and dimensions of backing sheet and sterility as well as the acceptable range for each item and an expiry date.

All the starting materials must be recorded, with source and batch numbers and samples of each analysed and kept. For each batch there must be a manufacturing record describing every stage of the manufacture of the product, including recorded in-process measurements or observations. The staff responsible for each of the stages of manufacture must sign and date these records to certify the completion of the manufacturing stage. This record must be kept with a Quality Control record which details the analyses of in-process and final product samples.

Instructions for performing all operations must be written down and displayed. This also includes cleaning, wearing the appropriate clothing for the manufacturing area, environmental control, product sampling and testing and equipment operation. This includes large items of equipment, e.g.: centrifuges and small items such as weighing balances and pH meters. Service histories and calibration records for all items of equipment must be kept.

All changes in the process, e.g.: addition of a new additive or a different concentration of additive, must be documented and validated.

8.4.1.4. Quality control

Quality control ensures that product specifications have been met, sampling procedures are in place and that a product is not released without satisfactory testing.

All materials used in the process must be tested, as well as intermediate products and the final product. For this process this would require testing of the initial starting plasma and

cryoprecipitate, PEG, dissolving buffers, water, the final PEG protein precipitate, urea, any additive used, the coagulants - hydrochloric acid and calcium chloride, dehydrating agents such as acetone and the final product. Routine testing would be carried out in house whilst more complex measurements, such as the cell binding properties of the material or examination of the fibre orientation, might only be made for the first few batches and then from time to time to ensure reproducibility and would be contracted out. Samples of each starting material must be kept along with the batch records for at least two years after product release whilst samples of the final product should be kept for at least one year after the expiry date, if stability permits.

8.4.1.5. Complaints and product recall

If a complaint is received it must be reviewed. It should be possible to access the batch records using a code number on the product in question and if necessary locate and recall all other fibres made in that batch. The results of such an investigation should be recorded and implemented appropriately.

8.4.1.6. Self inspection

The entire process should be inspected every three months to ensue that GMP is being implemented and that the process complies to the appropriate guidelines.

8.4.2. Fibronectin / fibrinogen preparation

The production of the fibronectin/fibrinogen precipitate would be contracted out to the blood transfusion service (BTS). Those providing the contract are responsible for ensuring that the material is manufactured to specification by a GMP regulated process. It is essential that the batches of precipitate are checked by a Qualified Person before their release from the BTS and the protein composition is within the range specified for the product.

The BTS already ensure that their plasma collection from the donor and cryoprecipitation complies to GMP standards so only GMP standard enrichment of fibronectin would need to be put in place. The final fibres must be traceable to the

original plasma donations using batch records and appropriate documentation. Access to these documents will be required in the event of a product complaint or recall. This is particularly important with respect to virus testing of the original plasma donations used to make the fibronectin fibres.

8.4.3. Wet spinning

The wet spinning rig should be situated within a clean area and clearly labelled with the batch and fibre type which is currently being spun. It should be possible to disassemble the rig for cleaning purposes and when reassembled it should operate as it did before. The dope reservoir, coagulating and washing tanks should be autoclavable. The pump delivering the dope should be calibrated for each batch used and the viscosity of each batch of solution should be measured and recorded. Any additives used should be of a suitable grade for the production of pharmaceutical products and the coagulating and washing solutions should be sterile. Alginates which have been approved by the FDA for use as wound dressings and as food additives are available at a suitably high grade (Shapiro and Cohen, 1997). A appropriate grade urea must also be chosen although this may only be available at high cost. The formed fibres should finally be collected and washed before being passed to an area with a higher cleanliness rating for drying and packaging.

8.4.4. Drying and packaging

If a freeze dryer is used to remove liquid from the fibres, drying times and temperatures must be optimised and the process validated to give a constant final moisture content. If the fibres are to be air dried, air filters should be used to sterilise the incoming air and the air entry and exit vents should be situated so that 'used' air does not pass across the final product.

The use of backing materials to support sheets of fibres has been discussed. Collagen sponges, bought in from other manufacturers, would have to be tested in the same manner as other starting materials and details of their supply and batch number recorded with the details of the final product. Sterility is particularly important at this stage.

The individual fibres or fibres on sheets should be packaged and gamma irradiated to ensure sterility. The irradiation should be done in batches with details of each batch recorded and validation should be in place to ensure that the intended dose is delivered to all the product. It is important not to rely solely on terminal gamma irradiation for sterility, the entire process must be designed with sterility in mind.

The addition of cells to sterilised products should also be performed under clean conditions. Donor cells should be checked for infective agents and cells from different donor sources should not be mixed. All cells should be clearly labelled with source, date of first culture and passage number. Media components should be tested by the Quality Control department to ensure consistency and lack of infection. All cell work should be carried out in a geographically distinct location to the other manufacturing operations.

The finished products should be labelled with a final batch number and marked with a more recent expiry date than the known product stability. Products with added cells would probably be made on demand, and would therefore have a short expiry date.

8.4.5. Distribution

Final products should be stored at either 4°C (fibres only) or frozen (cells and fibres) until distributed. Records of the destination of each batch must be kept. The buyer is responsible for keeping track of which products enter which patient but if a complaint is made the batch number will be required to trace the manufacturing process back to the initial plasma donor. All products should be discarded after their expiry date and products for laboratory use or clinical trial use should be marked accordingly.

Detailed and unambiguous instructions concerning product use, rehydration or thawing and stability in the body should be provided with each product distributed.

8.4.6. Viruses and prions - inactivation / removal issues

There are a number of infective agents which are potentially transmissible by blood and blood products. These include viruses (section 8.4.6.1.), bacteria causing sepsis and perhaps prions (section 8.4.6.2.), although data for this is at present inconclusive. The possibility of infection can be limited by restricting the choice of donor, screening all donations for infective agents and including inactivation / removal steps in the process (Rutter, 1994). There is a preference for virus inactivation steps above removal procedures, such as chromatography, because the assurance of virus removal by surface charge is less than inactivation by a denaturing agent (Berthold and Walter, 1994).

The cryoprecipitate used for this work originated from a pool of closely monitored donors providing plasma for special products e.g.: anti-Rhesus (D) and anti-Hepatitis B. These donors, like all others, are screened for HBsAg (the hepatitis B antigen), anti - HCV (hepatitis C virus), anti-HIV1 and HIV2 (human immunodeficiency virus) and syphilis serology, however, the pool of plasma that is created from their donations is much smaller than for other plasma products (200 litres compared to many thousands of litres (Foster and Cuthbertson, 1994)), reducing the probability of infection. This pool of plasma, although regarded as safer than the general pool, would not be large enough to support large-scale production of fibronectin/ fibrinogen fibres. It would therefore be advantageous if other fibronectin / fibrinogen sources within the plasma fractionation industry could be exploited and this requires a review of virus inactivation procedures. The production of plasma products generally requires there to be two validatable virus inactivation steps

8.4.6.1. Viruses

The main viruses which are known to be transmittable by blood and blood products are listed in Table 8.2. They are divided into two subsets - those with a lipoprotein envelope and those without. The presence or absence of this envelope appears to determine the resistance of the virus to any particular inactivation procedure. The effectiveness of any virus inactivation step must be validated by using model viruses with similar size and structure to the human virus. Validation of all virus inactivation techniques is essential and normally carried out by scaling down the manufacturing process and monitoring the removal of a known, added quantity of infectious material (Foster and Cuthbertson,

1994). Virus size will control the success of removal techniques such as filtration with the effectiveness of any inactivation step being measured by the reduction in the log. number of viruses / mL.

Virus	Size (nm)	Lipoprotein envelope	Found in blood	Found in blood derivatives	Donor viraemic frequency	Model virus for validation
HBV	42	Yes	Yes	Yes	1/100 000	Duck HBV
HIV-1	110	Yes	Yes	Yes	1/500 000	Simian IV
HCV	35	Yes	Yes	Yes	1/3 300	Sindbis
HAV	30	No	rare	No	<1/10 000	Polio
HDV	NA	Yes	Yes	Yes	NA	NA
Parvovirus B19	24	No	Yes	Yes	1/10 000	Canine parvovirus

Table 8.2. *Summary of viruses potentially transmittable by blood and blood products. Data taken from Prowse (1994) and Rutter (1994). NA - data not available. Viruses which are now becoming the standard models for inactivation studies in Europe include Bovine Viral Diarrhoea Virus for Hepatitis C and Pseudorabies for Hepatitis B. For Hepatitis A and HIV the actual human virus is used.*

Tested methods of virus inactivation are summarized in Table 8.3 and demonstrate the difficulty in combining effective removal of the infective load with preservation of protein activity. The solvent / detergent method is becoming the first choice method for treating plasma and plasma products. The combination of solvent and detergent attacks lipid in the envelope of HIV, HCV and HBV and leads to either total virus disruption or disruption of the cell-receptor recognition site (Horowitz, 1989). Tests on the virus inactivation of fibrinogen and fibronectin spiked with Sindbis virus revealed that 0.3% tri-(n-butyl) phosphate (TNBP), an organic solvent, and 1.0% Tween 80, a non-ionic detergent, resulted in a viral clearance of 5.5. and > 5.4 logs after 1 hour for fibrinogen and fibronectin respectively. The action of TNBP/Tween 80 did not affect the electrophoretic migration rate of either protein, however, heating to 60°C, as a model of pasteurisation, did. There was little or no change in the protein's functional activity (Horowitz, 1989). Binder and Nemeth (1988) describe the production of a virulent-free,

injectable fibronectin solution which has been subjected to two heat treatments, both at 60°C for 10 hours. Fibronectin, like many other proteins, had to be stabilised with at least one additive, usually a low molecular weight sugar or amino acids, before heat treatment (Ben-Hur and Horowitz, 1996).

Approach	Blood component	Advantages	Disadvantages
Wet heat	Purified proteins Plasma	Easy to carry out All viruses susceptible	Recovery of protein activity is moderate Non-enveloped viruses killed to a lesser extent May need to remove stabilising agents after treatment
Dry heat	Purified proteins	Performed in final container High protein recovery Easy to carry out and validate	Non-enveloped viruses are not completely killed
Solvent-detergent	Purified proteins Plasma	Enveloped viruses very sensitive Recovery of protein activity is close to 100% In process monitoring easy	Non-enveloped viruses are not inactivated Plasma must be pooled Need to remove agents
Nanofiltration	Purified proteins	Easy to add to existing process	Limited to proteins of lower molecular weight
UVC light	Purified proteins Plasma	Inactivates all virus types	Rutin must be added to protect protein activity

Table 8.3. *Advantages and disadvantages of the different approaches for virus inactivation of plasma and plasma products (adapted from Foster and Cuthbertson, 1994; Prowse, 1994 and Ben-Hur and Horowitz, 1996).*

Techniques such as gamma irradiation have been disregarded as a method of sterilisation due to the high doses required and the resulting damage to the protein (Hiemstra, 1991). The use of 8 M urea for 4 hours at 37°C has been shown to inactivate 5 logs chimpanzee

HBV/mL without significantly altering the antigenicity of the final HBsAg product (Tabor, 1983).

Terminal dry heat treatment has not been tested on fibronectin / fibrinogen fibres but there have been successful results reported for treatment of plasma products. A combination of solvent/detergent steps and terminal dry heat treatment was shown to cause a reduction of $\geq$ 10.2 logs of a HCV model virus in a Factor VIII concentrate and a combination of dry heat treatment and freeze drying reduced the animal parvovirus (non-enveloped) load by at least 10.3 logs (McIntosh et al, 1998).

The use of a solvent-detergent treated feedstock would be ideal for the production of fibronectin / fibrinogen fibres, if the cell binding domain of the protein remains unaffected. Myers *et al.* (1991) described the removal of TNBP / Tween 80 by precipitation and washing steps further down the protein extraction process as sufficient to be within regulatory limits for the two components. Since this solvent/detergent treatment is a technique which is already in place for the production of other protein products, e.g. Factor VIII, there would be no need to develop a new inactivation process. Other possible virus inactivation steps which could be included in the wet spinning process are the use of high concentrations of urea, described earlier, or acid treatment. Incubation at pH 4.25 for 24 days has been shown to inactivate viruses, (Rutter, 1994) however fibronectin / fibrinogen mixtures would precipitate at this pH. The very low pH in the coagulation bath may act as a virus inactivation step although the effectiveness of such a technique is difficult to validate in a solid fibre. Terminal dry heat treatment of the final fibres should also be evaluated.

8.4.6.2. Prions

Previously all concerns about transmissible agents in blood have surrounded viruses. The advent of a new variant of the neurodegenerative disease, Creutzfeldt-Jakob disease (nvCJD), has turned attention to the detection and removal of the causative prions. The disease causing prion protein, named PrP^{sc}, is a protease resistant form of a normal cellular protein, PrP^{c}. At present most research work uses the normal protein, PrP^{c}, which can be detected in plasma and blood, or spiking with scrapie as a model to test techniques for removal of the abnormal form (MacGregor *et al.*, 1998). There has yet to

be demonstration of transmission of the sporadic form of the disease through blood products however the differing properties of the variant form may cause it to act differently. This new variant form, which accumulates in the neural and lymphoid tissue, is fibrillar and insoluble (Mabbot, 1998). This low solubility and its ability to adhere to surfaces and form aggregates may make it susceptible to removal by some of the existing precipitation and adsorption steps currently in place in the plasma fractionation process (Foster, 1998). Studies of the reduction of PrP^c or scrapie in the processes used by the Protein Fractionation Centre, SNBTS, to produce fibrinogen and Factor VIII concentrate showed a 12 and 8 log. reduction respectively in the model agent during production.

Urea has been tested as a prion inactivation agent in the growth hormone (HGH) purification procedure. Using an 8 M urea treatment was found to remove scrapie infectivity from the HGH preparation and the use of concentrations of at least 6 M urea has been suggested as a prion inactivation step (Dormont *et al.*, 1989).

8.5 Experiments with potentially virus inactivated material

Virus inactivation is an important topic if pooled human plasma products are to be used to manufacture medical devices. Preliminary work exploring the use of virus inactivated feedstocks to produce fibronectin materials are now described.

8.5.1. Use of heat pasteurised fibronectin / fibrinogen

Batches of fibronectin solution were heat pasteurised for 10 hours at 60°C with 20% (w/v) Sorbitol to protect the protein from the effects of the heat (MacLeod *et al.*, 1983). To test the effect of heat pasteurisation on cable drawing, experiments similar to those described in section 4.2, where salt or acid was added to the solution, were repeated. Fine protein strands could be drawn from a purified, pasteurised fibronectin solution, if sorbitol had been added prior to heating. However without sorbitol no fibronectin strands could be drawn. On the addition of acid to a sorbitol protected, non-purified fibronectin/fibrinogen mixture a precipitate was formed which did not aggregate to form cables but again fine fibronectin strands could be drawn. Removal of the sorbitol by

dialysis into a phosphate-citrate buffer did not aid cable formation in pasteurised solutions. Thus the addition of 20% (w/v) sorbitol is necessary to protect fibronectin during pasteurisation, however higher concentrations may be required to protect the fibrinogen, essential to cable drawing.

8.5.2. Using solvent /detergent treated fibronectin / fibrinogen

The column breakthrough fraction from Factor VIII production contains both fibronectin and fibrinogen and has been solvent / detergent treated during the process. Spinning trials showed that this virus inactivated feedstock, containing 60% fibronectin and 40% fibrinogen, could be used to spin protein fibres on a pilot scale. However the effect of the solvent/detergent treatment on the cell adhesion properties remains to be determined.

8.6. Summary

The aim of this chapter was to outline a potential process for the manufacture of fibronectin based tissue engineering scaffolds and to consider Good Manufacturing Practice, regulatory and virus inactivation issues with respect to the chosen process.

Process flowsheet: An overall process has been developed, however the final application of the finished fibres or fibre will affect the dope formulation used, the size of fibre made and the method of fibre collection and drying implemented. The need to incorporate cells into the final product will also be determined by the final use of the fibres.

Regulatory issues and GMP: A globally agreed approach to regulation of tissue engineered products is still to be organised. Products such as the fibronectin fibres would probably be regulated as a biological / blood product by Britain and as a combination product by the US. The addition of cells, including autologous cells, increases the number of rules and guidelines to be applied. Good Manufacturing Practice is essential to produce a product of a suitable grade for clinical trials. One important feature of GMP is that the final product used in a surgical procedure must be traceable back to the original plasma donor by detailed documentation.

Virus inactivation: There are a number of virus inactivation steps which could be included in the process, including solvent / detergent treatment of the feedstock, low pH treatment and high concentrations of urea. These steps would potentially cover the infective agents currently known. Initial experiments with solvent / detergent treated feedstocks look promising. Optimisation of a heat pasteurisation step could be carried out with an increased percentage of a protective agent, to prevent fibrinogen denaturation and the effect of all virus inactivation steps, including possibly terminal dry-heat treatment should be tested with respect to the material's cell adhesion properties.

9.0. Discussion and conclusions

9.1 Discussion

The interdisciplinary field of tissue engineering has developed due to perceived problems with currently practised techniques. These include long term failings of surgical reconstruction and limited functions of mechanical devices and drug therapy combined with a shortage of organ / tissue donors (Peters and Mooney, 1997).

In order to develop a satisfactory replacement tissue, the environment of cells in a non-injured tissue must be understood. The degree of mimicry required between an 'engineered' tissue and the natural tissue will depend upon the role of the replacement tissue. Tissue replacements can thus be either simple scaffolds or highly complex, metabolically active structures. The fibronectin - based materials discussed here are scaffolds to provide cells with directional cues. They have potential for uses other than skin replacement and future prospects as a base for forming more complex products.

The production of fibronectin-based materials for orientated tissue repair has been considered with respect to protein conformation and interactions, cell adhesive properties and material properties. Potential safety and regulatory issues have also been examined.

9.1.1 Summary of materials made

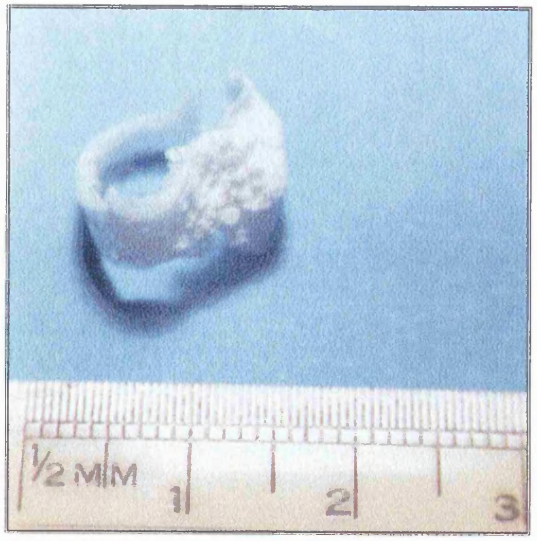
The first fibronectin materials were mats made by concentrating a purified fibronectin solution in an ultrafiltration cell at the same time as applying a unidirectional shear (Ejim *et al.*, 1993). The formed mats had dimensions 1 x 2 cm, were several millimetres thick and consisted of a network of 5-10 μm diameter, orientated fibronectin fibrils. These fibronectin mats have been shown to support the attachment of keratinocytes (Prajapati *et al.*,1996) , rat tail tendon and human dermal fibroblasts *in vitro* (Ejim.*et al*,1993). *In vivo* experiments have demonstrated successful use in both nerve (Whitworth *et al.*, 1995a) and dermal repair (Brown *et al.*, 1998) as well as depots for growth factors (Whitworth *et al.*, 1995b, 1996). The advantages and disadvantages of the product and manufacturing process for the fibronectin mats and other fibronectin materials are given in Figure 9.1.

The second fibronectin materials produced were 'cables'. These were drawn upwards from a precipitate of two plasma proteins, fibrinogen and fibronectin, following the addition of acid to a cryoprecipitate-derived solution. Cables drawn were between 100 and 500 μm in diameter and up to 20 cm long. They have been shown to support the attachment and alignment, *in vitro*, of human dermal fibroblasts (Harding *et al.*, 1998), Schwann cells, (Ahmed *et al.*, 1998d), endothelial cells (Harding, S.I., personal communication) and human tendon fibroblasts (Underwood, unpublished results). Proposed methods of using these materials include attaching the cables to a backing material or the use of bundles of the cables.

Spun fibronectin fibres were made by extruding a high concentration fibronectin / fibrinogen mixture through a fine orifice into an acid and salt coagulation bath. The diameter of the final fibres depended on the diameter of the orifice used for extrusion and the drawing treatment of the fibres after formation. Fibres with diameter 350 μm up to 1500 μm have been spun (Ahmed *et al.*, 1998b) on a small scale whilst on a pilot scale 500 μm diameter fibres have been produced. These fibres are reported to support, *in vitro*, the attachment and alignment of Schwann cells (Ahmed *et al.*, 1998b), endothelial and dermal fibroblasts (Harding, S.I., personal communication).

9.1.2 The potential role for fibronectin and fibrinogen materials as tissue engineering scaffolds

Fibronectin and fibrinogen are found naturally in healing wounds. Both plasma proteins are detected during epithelial migration in deep dermal wounds, during the early stages of wound repair. Cellular migration of fibroblasts, epithelial cells and macrophages is known to be promoted by fibronectin during natural healing and fibroblasts in wounds tend to align along the same axis as fibronectin fibrils in the matrix. Newly produced collagen has also been seen to align along the same axis as the fibronectin (Colvin, 1989). This combined with the role fibronectin plays as a nucleating agent, binding cells, extracellular matrix components and fibrinogen and fibrin during the blood clotting process, make fibronectin and fibrinogen ideal components for the formation of a natural tissue repair material



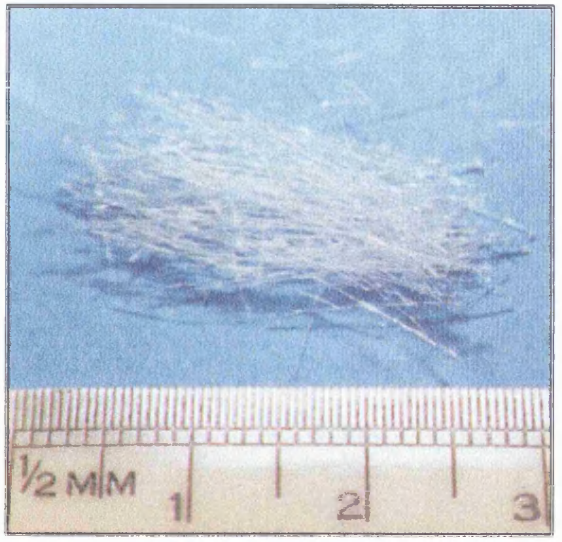
Fibronectin mat:
Advantages: Mat has thickness and can be applied directly to wound, to bridge a gap or used in layers

Pure fibronectin provides excellent cell attachment and mat has proven cell orientating ability. Has been tested as a nerve conduit and a dermal repair material *in vivo*. Can be used as a depot for growth factors or as a raft for cell transplantation.

Disadvantages: reduced cell migration due to high degree of cell attachment.

Fibronectin remains in solution after manufacture.

Difficult to scale/ control/ validate process



Drawn fibronectin cables:
Advantages: No affinity chromatography step required - reduces time / cost and validation requirements of process.

Easy to produce

Good cell orientation / migration properties

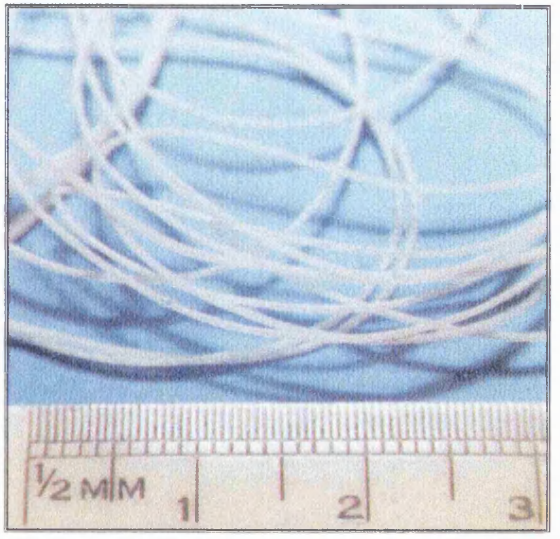
Flexible uses - as bundles of fibres or attached to a backing material

Can be drawn down to a fine diameter when wet

Disadvantages: Difficult to scale / control / validate

20% protein remains in solution following pptn.

Final product is brittle when dry and difficult to handle.



Spun fibronectin fibres:
Advantages: No affinity chromatography step required - reduces time / cost and validation requirements of process.

Technology is scaleable and validatable

Flexible manufacture - fibres can be a wide range of diameters (<500 -1500μm)

Can alter composition of feedstock and add additives to alter the final material properties.

Little waste on formation

Disadvantages: High viscosity / high concentration solutions required. Use of certain solvents / coagulants may affect cell binding properties

Figure 9.1. *Advantages and disadvantages of fibronectin materials and their production processes*

The addition of topical fibronectin to corneal wounds has been used to monitor the effect of applying exogenous fibronectin on wound healing. Hynes (1990) reported that there was only slightly improved corneal healing with added fibronectin compared to natural healing. Consequently, he reported no significant advantage to applying topical fibronectin, unless there is an endogenous shortage. However in some wounds, such as chronic non-healing ulcers, there is a great deal of proteolytic activity, including breakdown of fibronectin. Thus the addition of fibronectin materials may enhance repair in these cases, in combination with treatment of the underlying causative conditions. The creation of 3-dimensional scaffolds from fibronectin would add a new perspective to wound healing, not provided by topical fibronectin application. Scaffolds used in tissue engineering aim to provide a support structure for anchorage-dependent cells and a matrix for their localised implantation into the body (Langer and Vacanti, 1993). The development of many matrices has centred around the use of collagen or synthetic polymers and in some cases there has been interest in combining the excellent cell binding properties of fibronectin with the tensile properties of the synthetic materials (Barrera *et al.*, 1993). Recombinant proteins are also being developed, combining the cell adhesion sequences of fibronectin with the tensile properties of silk (Cappello and Crissman, 1990; Anderson, 1994).

Fibronectin and fibrinogen are degraded by the body. During natural wound healing, levels of fibronectin and fibrinogen in the wound are depleted 7-14 days after the initial injury. Consequently, scaffolds made from these proteins may have to be stabilised if they are to act as a directional guidance cue for wound repair (section 9.1.5). It is important that in stabilising the material it does not become insoluble, as excess fibronectin can lead to excess collagen deposition. fibrosis and inflammation (Hynes, 1990).

In the manufacture of fibronectin and fibrinogen materials, knowledge of the effect of environmental conditions on the proteins has been used to manipulate their molecular conformation. In doing this it was important not to affect the natural roles the proteins play in wound healing.

9.1.3. The fibronectin feedstock

For fibronectin mat manufacture, fibronectin solution purified by affinity chromatography was required. The feedstock for this process had traditionally originated from a glycine supernatant fraction of the Factor VIII extraction process, Figure 1.1 (BPL, Elstree, UK). Solutions derived more directly from cryoprecipitate (PFC, Edinburgh, UK) were also purified and could be used to produce fibronectin mats. Urea, used to elute fibronectin from the chromatography column, was believed to play an important role in opening out the fibronectin molecule into an extended conformation (Markovic *et al*, 1983), allowing inter-molecular interactions to occur. High salt concentrations are also reported to promote the formation of an extended conformation (Williams *et al.*, 1982; Tooney *et al* ., 1983; Sjoberg *et al.*, 1989) . When a fibronectin solution was eluted from a Heparin-Sepharose column using 4 M NaCl this solution could also be used to form fine fibronectin strands suggesting that fibronectin fibril formation during mat preparation was a feature of fibronectin in its extended form.

Affinity chromatography, on a large scale, is an expensive and time consuming unit operation. To bypass this step, methods of manufacturing materials from non-purified fibronectin / fibrinogen solutions were examined. Both fibronectin and fibrinogen are contained within cryoprecipitate and are a byproduct of the validated Factor VIII extraction process, with a solvent/detergent virus inactivation step already in place. For this research, cyroprecipitate from small plasma pools from closely monitored donors was used. For large scale production of tissue repair materials, feedstock derived from large plasma pools would be required and so validated virus inactivation steps must be included in the process. In all cases it would be essential to test whether these virus inactivation steps affect the material's cell binding activity. Potential treatments of the feedstock include solvent-detergent treatment or pasteurisation of the protein feedstock after the addition of protein stabilisers (Foster and Cuthbertson, 1994; Prowse, 1994 and Ben-Hur and Horowitz, 1996). Other treatments can be included in the material manufacture process or treatment of the final product.

The mat making process used only purified fibronectin. However, data described by Palecek *et al.* (1997) revealed that high concentrations of fibronectin may assist cell adhesion but reduce cell migration by tightly binding the cell to the fibronectin substrate. Using fibrinogen to effectively 'dilute' the fibronectin's binding functions increased the

cell migration rates along fibronectin fibres, essential for wound repair (Ahmed *et al.*, 1998c).

Production of drawn cables required the formation of both hetero-and homo - polymers of fibronectin and fibrinogen. These were produced by the precipitation of the two proteins at reduced pH. Interactions between the two proteins were believed to occur in solution because the pH of minimum solubility of both proteins in solution was found to be 2 pH units below literature values for the individual proteins. Boughton and Simpson (1984) have reported differences between the isoelectric point of purified fibronectin solutions and fibronectin in plasma, therefore there is potential for heterogeneous interactions to alter pH-dependent solubilities. Measurement of protein in solution demonstrated that both proteins formed the cable in a ratio approximately that in solution.

Since results have shown that cell migration rates are dependent upon the composition of the substrate, it may be important to consider the composition of natural migration matrices, such as the fibrin clot in wound healing. Fibronectin is the major non-fibrin protein in the clot and can constitute 4-5 % of the mass of a plasma-derived clot (Moon and Kaplan, 1989). The repair of other tissues may have healing mechanisms with differing protein compositions. It would therefore be important to balance the fibronectin concentration to achieve both good cell adhesion and cell migration. Since the feedstocks are prepared by a PEG fractionation method, differing protein compositions should be achievable. Indeed, without intention, a feedstock with only 25 % fibronectin and 75 % fibrinogen was prepared compared to the usual 65 % fibronectin and 35 % fibrinogen. Optimisation of the fibronectin enrichment process is required to achieve the preferred ratio. For both the manufacture of cables and fibres, the fibronectin / fibrinogen feedstock was dissolved directly into a solvent. It would therefore be easy to accommodate changes in the initial feedstock into the process.

9.1.4 Process design

The production of fibronectin mats in the enclosed system of the ultrafiltration cell worked well as a small-scale, batch operation although it was difficult to regulate and control the production of identical, orientated mats. The system resulted in one mat

being formed every 15-60 minutes on the cell's stirrer shaft, depending on the solution protein concentration. After the formation of one mat, the cell had to be opened and the stirrer shaft replaced ready for the formation of a new mat. Thus the advantage of the enclosed system was lost and the risk of contamination increased. Also, the solution fibronectin concentration decreased as mats were formed and the system had to be opened to allow solution replenishment after the production of 3-4 mats. To scale this system up would be complex. To produce larger mats both larger diameter shafts and cells would be required, in order to maintain the current system geometry. To increase the number of mats produced a system with many shafts operating in a large cell would be required. This would eliminate frequent changing of the stirrer shaft and the associated contamination risk, however it would pose a design challenge to prevent the turbulence from each shaft interfering with the next. A continuous flow system, maintaining the fibronectin concentration, would also be required. As a result of these considerations it was decided that this would remain as a small scale process and a different approach would be adopted for the larger scale production of fibronectin materials.

The manipulation of the fibronectin molecule into an extended conformation by urea was used during the fibronectin mat manufacturing process. To produce the drawn fibronectin cables, the molecules were again manipulated into their extended conformation but using low pH. This phenomenon has been extensively reported for fibronectin (Williams *et al.*, 1982; Tooney *et al.*, 1983; Markovic *et al.*, 1983b; Sjoberg *et al.*, 1989; Benecky *et al.*, 1991) and fibrinogen (Esouf, 1972). Cables were drawn from solutions after the reduction of the pH to 4.0-4.5, a pH greater than that seen from solubility curves as that of minimum solubility. This allowed the precipitate to be drawn without the extensive aggregation of precipitate which was seen at pH 3.0 and removal of protein from solution resulted in further protein precipitation, until 80 % of the protein had been precipitated from solution. Markovic *et al.* (1983b) describe excessive precipitation of fibronectin between pH 3.5 and 6 for fibronectin concentrations 0.08-1.5 mg/mL. Decreasing the pH further to pH <1.0 led to extensive protonation of the molecule and disruption of probably both intra and inter-chain bonds leading to protein aggregation.

The action of drawing the cables from solution orientated the fibronectin and fibrinogen molecules and allowed them to form intermolecular bonds. As a result the cables had a high tensile strength (61 N/mm^2) but were brittle, both characteristics of highly orientated materials. Drawing is not a technique suitable for large scale manufacture - it is difficult to control the diameter of the cable and the amount of cable produced. To ensure homogeneous acid addition a stirred system could be used with a low, < 10 rpm, stirrer speed. However this would encounter similar problems to the stirred fibronectin mat manufacture as the formed cables would collect around the stirrer shaft.

To wet spin protein fibres, a high concentration, high viscosity protein dope was required. Urea was chosen as a solvent because it had been used successfully in the mat making procedure and is used at high concentrations to elute fibronectin from affinity chromatography columns. Some of the regions not stabilised by disulphide bonds have been found to be at risk from denaturation by high concentrations of urea (Markovic *et al.*, 1983) and this includes the cell-binding regions. Fortunately, *in vitro* tests of materials spun from urea based dopes demonstrated that the fibronectin still retained its cell-binding activity (Harding, S.I.; personal communication, Ahmed *et al.*, 1998b). It was necessary to choose a solvent which promoted the molecule's extended conformation to enhance molecular aggregation into linear polymers on extrusion. Using 6 M urea, solutions of protein concentration 100-140 mg/mL could be made. These however had relatively low viscosities (98 mg/mL had viscosity 55 mPa s, 0.55 P) compared to values quoted for spinning protein fibres, 25-380 P (Young and Lawrie, 1974). Use of a different solvent could result in the production of higher viscosity dopes, although the cell binding activity must not be destroyed in the dope preparation process. Guanidinium hydrochloride has been used at lower concentrations than urea, as a 'denaturant' and is available as a high grade reagent, however it can have drastic denaturing effects on protein (Khan *et al.*, 1990) and requires implementation of measures to ensure operator safety. Traill (1945) described the use of sodium hydroxide as a solvent, in place of urea, and Young and Lawrie (1974) used sodium hydroxide and acetic acid to prepare viscous spinning dopes. Swingler and Lawrie (1977) added 40 % NaOH directly to plasma to spin porcine plasma. Evidence in favour of the use of urea is its potential to act as a virus / prion inactivation step (Tabor *et al.*, 1983; Dormont *et al.*, 1989).

To spin protein only fibres on a small scale from a syringe, a solution with minimum 70 mg/mL total protein, was required. The protein composition used for these experiments was 65 % fibronectin / 35 % fibrinogen and the solution had apparent viscosity 20 mPa s (0.2 P). Hypodermic needles were used to simulate one-holed spinnerets, with a path-length 1 cm and diameter 350 μm. The mass average shear rate, γ_{av}, for this system was calculated as 31700 s^{-1}, using equation 6.2. Since these protein only solutions were not shear thinning, the apparent viscosity remained at 0.2 Poise, even at this shear rate, and a continuous flow of dope coagulated into fibres. When spinning using the pilot scale rig, the viscosity of the solution needed to be higher than for small scale production. Increased intermolecular interactions were required for the continuous coagulation of fibres. On extrusion through the multi-holed spinnerets, typical of the textile industry, solutions of such low viscosity did not form a continuous stream on extrusion and did not coagulate into continuous fibres. The shear rates for these spinnerets were high, 6300 - 39300 s^{-1}, and the path lengths too low to orientate the molecules, in preparation for coagulation. The addition of alginate or carboxymethylcellulose to the dope, increased the apparent viscosity above that of a protein only solution at high shear rates and formed a continuous thread on extrusion. An 88 mg/mL total protein solution with either 1 % alginate or 0.5 % CMC had an apparent viscosity of 160 mPa s at a shear rate of 1000s^{-1}, whilst for a 98 mg/mL protein only solution, the apparent viscosity was 55 mPa s.

Calculation of a solution's viscosity before spinning, knowledge of the protein concentration and a study of shear imparted to the fluid by the spinneret allows a prediction of whether solutions will form fibres. For example, to spin fibres of final diameter 250 μm, a spinneret of diameter 500 μm is used and it is assumed that drawing the fibre after formation will reduce the diameter by half. The mass average shear rate of this spinneret would be 10900s^{-1}, eight times greater than that for a 1 mm diameter spinneret. Therefore solutions chosen for spinning fine fibres must have an apparent viscosity at this higher shear rate equivalent to the apparent viscosity at 1000s^{-1} of solutions which have been used to successfully spin fibres (413 mPa s for 140 mg/mL protein , 1 % alginate). For low viscosity solutions, applying a low shear rate to a solution over a long duration, i.e.: using a long capillary of 1 mm diameter, appeared to work best. From experimental observations it appears that the composition of the

protein feedstock may alter the final viscosity of the solution. Solutions with high concentrations of fibrinogen are believed to have a higher viscosity than those with high levels of fibronectin.

Altering the method of dissolving large quantities of protein precipitate may remove the need for viscosity enhancers. However additives can affect the final properties of the product (section 9.1.5). Alginate has been used mainly as a wound covering rather than a repair scaffold with cell adhesion functions. However, new applications of alginate are being described with functions in cell transplantation (Shapiro and Cohen, 1997) and in an oxidized form as a cell scaffold (Bouhadir, *et al.*, 1998). Nerem and Sambanis (1995) describe the use of high purity alginate with a high glucuronic acid content to reduce inflammatory responses. Other additives such as sodium carboxymethylcellulose and dextran are also available in high quality grades and have been approved for food and medical use.

The coagulation step requires that the solvent, in this case urea, is removed and that the protein is precipitated. Fibronectin and fibrinogen molecules will be in their extended conformations and the protonation, caused by the low pH conditions in the acid bath, results in a certain amount of denaturation, leading to molecular aggregation. Orientation of the molecules in the capillary prior to extrusion assists the overall fibre formation process.

Industrial wet spinning of proteins typically precipitates out the protein around their isoelectric point in the coagulation bath. Small scale tests to spin fibres into a coagulation bath with pH equal to the minimum solubility resulted in the production of fibres which were weak, often not discrete and difficult to handle. Improved fibre formation was found at very low pH < 1.0, where some denaturation of the molecule occurs. These poor results around the pH of minimum solubility may have been due to the low viscosity / low concentration solutions used. Increasing the solids content (protein concentration in solution) will increase the number of inter-molecular interactions at the point of minimum solubility and enhance precipitation and fibre formation. Denaturation, at pH < 1.0, is an extreme process and will precipitate the protein irrespective of concentration. Acid / salt combinations were chosen for the coagulation bath, in preference to acid alone, to minimise the concentration of acid

required and reduce the possibility of acidic hydrolysis of the protein. Inclusion of salt also assisted in dehydrating the fibre, by drawing water from inside the filament. A coagulation bath containing calcium chloride was used to spin fibres containing alginate, due to the formation and consequent precipitation of insoluble calcium alginate from soluble sodium alginate.

Washing steps are required to remove the often aggressive chemicals from the fibre which could affect cell viability. Phosphate buffered saline has been used successfully for fine fibres, however washing with tris buffers has proved much more successful at neutralising the pH for fibres with diameter greater than 2 mm (Harding, S.I., Ahmed, Z., personal communication). If it is essential to remove the alginate from the fibres, before use, then a sodium hydroxide washing step could be considered. Immersion of the fibres in NaOH, results in the formation of soluble sodium alginate (Rogers, V., personal communication). However, the removal of alginate will have an effect on the fibre's final properties. The fibres spun on a pilot scale were dehydrated at room temperature in acetone. It was found that fibres that had not been dehydrated had lower tensile strengths than those which had been treated. Concern surrounding denaturation of the proteins by this solvent treatment could be reduced by using chilled ethanol as a dehydrating step. The use of chilled solvents is the method used in the plasma fractionation industry to prevent protein denaturation during Cohn fractionation (Kistler and Friedl, 1980).

Fibres were drawn to a finer diameter to increase their orientation. Drawing fibres, by setting one set of rollers to rotate faster than rollers picking the fibres up from the wash bath, is a technique for increasing the orientation of the component molecules. All protein fibres have low wet strength, making collection by rollers difficult. However, high protein fibres could be drawn down by rollers (tensile strength 28 N/mm^2) whilst those containing more alginate could not (tensile strength 6.6 N/mm^2). This low tensile strength for the high alginate fibres was partially caused by air trapped in the filaments during coagulation and also poor inter-linkage of adjacent protein and alginate molecules in the fibre.

The flowrate used for the pilot scale work, 15 mL/min, was equivalent theoretically to the extrusion of 19 metres fibre/ min. through the 1 mm diameter orifice. With this

extrusion rate the fibres would only take 6 seconds to be drawn the entire length of the 1.8 metre coagulation bath. In practise, the fibres were extruded too quickly to be collected straight onto the roller and remained in the coagulation bath for longer than 6 seconds. The low wet strength of the fibres prevented the use of higher speeds for the collecting rollers. Immediately after extrusion the fibres appeared transparent, but turned white as coagulation occurred. Coagulation was complete when there was no further increase in opacity. Future fibre spinning trials would benefit from lower extrusion rates, allowing fibres to spend longer in the coagulation bath before removal.

The advantages of choosing a textile process for the production of fibronectin materials is that it is a truly scaleable process. Annual production of a casein fibre, 'Aralac' in 1943 was 5 million kg (Moncrieff, 1969) whilst at the opposite end of the scale small amounts of fibre could be produced using a 1 mL syringe.

As discussed earlier, large pools of fibronectin and fibrinogen would become available for use if appropriate virus inactivation procedures were put in place. From a 1 litre plasma donation, it has been estimated that 13 metres of drawn fibronectin cable can be made. Alternatively, from the same volume of plasma 1 metre of fibronectin and alginate fibre or 1.5 metres of protein only fibre can be spun. These yields have been used to calculate the amount of 200 μm cable or fibre that would be required to cover a backing sheet, using the techniques described in Chapter 7.0. The size of the sheet chosen, 5 x 7.5 inches (12.7 x 19 cm), is based on the size of sheets of Dermagraft-TC. To cover a backing material with cables or fibres spaced 1 mm apart, to promote cell orientation, would require 19 metres of cable or fibre, equivalent to 1.5 litres of plasma. Fibre spacing distances were chosen from work by Harding (1996). For the large scale production of these products, pools of plasma would have to be used. Burns patients must be protected from dehydration and therefore would be unable to donate the amount of plasma required to produce an autologous skin substitute. However, for patients with chronic ulcers, or for the production of other cell guidance conduits, (section 9.1.5) the patient's donated plasma could be banked and the appropriate composition fibronectin / fibrinogen precipitate prepared before wet spinning a suitable conduit. This removes the need for virus inactivation steps of the initial plasma, however rigorous testing will still be required because the plasma products will be banked and returned to the patient in a second surgical procedure.

The yield for the spun fibronectin / fibrinogen fibres appears much lower than for drawn cables because the spun fibres have a larger diameter but they can also be drawn down to finer diameters. The estimated volume of precipitated fibre / mg of protein in the dope is similar for spun fibres with and without additives as well as drawn fibronectin cables (range 2.6 - 4.0 x 10^{-3} cm^3/ mg protein in dope). Thus equivalent lengths of spun fibre, drawn to a finer diameter, can be produced from the same amount of protein as the fibronectin cables. In the former case, a very small volume of highly concentrated feedstock will be produced, whilst for cable production a larger volume of more dilute protein solution is prepared. To illustrate this, data from the preparation of batch 010 have been used to estimate the volume and concentration of each feedstock which can be prepared for 1 L of initial plasma donation. For the production of drawn cables, 39 mL of 4 mg/mL solution can be produced from 1 L plasma whilst in contrast, 1 mL of spinning dope with a total protein content of 150 mg could be made. There is believed to be less protein wastage during the spinning process.

9.1.5 Final products

The methods described above allow a great deal of flexibility in the amount and type of fibre which can be made. Cables on backing material, as potential dermal equivalents, have been described in this thesis. Large diameter fibres have been described as potential nerve conduits (Ahmed *et al.*, 1998b) and further research continues investigating the production of hollow fibres as vascular conduits (Harding, S.I., personal communication).

The properties of the final product can be altered in a number of ways. The balance between cell adhesion and migration can be changed by the fibronectin / fibrinogen composition of the material. Addition of alginate and increasing the protein concentration of the spinning dope has produced flexible fibres with evidence of permanent set and high extension at break, compared to the brittle cables drawn up from solution. The high tensile strength seen for the drawn, twisted protein cables (60.9 N/mm^2) was not seen for the spun fibres. Instead the average tensile strength for a spun, high protein fibre was 28.1 N/mm^2 comparable to 14.9 for a non-twisted, drawn protein cable. All the fibronectin materials were highly hygroscopic, similar to many protein

fibres, including wool, (Moncrieff, 1969) and could be drawn when wet.

Mats, cables and fibres all possessed ultrastructural orientation. The mats consisted of aligned 5-10 μm diameter strands, the cables were composed of micron-diameter fibrils and ridges of diameter 1-10μm could be seen on the surface of the spun fibres. This allows all the materials to provide cell guidance cues. There are many tissue repair functions where orientated repair is critical, including repair of peripheral nerves, tendon and blood vessel replacement.

The natural structure of dermis consists of a network of collagen fibres and therefore it is necessary to decide how accurately we need to mimic the uninjured tissue and how much the body should be left to remodel on its own. The results described in this thesis, demonstrating cellular attachment to collagen sponges may show enough orientation to give scar-reduced healing when layers of the material are inserted into *in vivo* wounds. However, with the production of the flexible spun fibres, it may be preferable to form networks of fibres, similar to those described by Langer and Vacanti (1993).

Nerves, tendons and blood vessels require orientation over a much smaller area, requiring only relatively short lengths of fibre to be spun. Conduits to replace these tissues are unlikely to require backing materials, instead just the fibre could be sutured into place. The choice of the final product would affect the properties of the fibre created. Schwann cells may have different fibronectin / fibrinogen requirements to dermal fibroblasts, however with the wet spinning system, changes in the dope can be made easily, although changes in viscosity need to be monitored. The flexibility of the system allows small diameter fibres to be spun. Larger fibres, up to 1500 μm (Ahmed *et al.*, 1998b) have been spun on a small scale. The final function of the fibre will alter the tensile properties needed. Fibres used in tendon replacement would require a high tensile strength and be required to promote the formation of extremely orientated, new matrix, whilst those for dermal repair need good elastic properties. The amount of additive, such as alginate or CMC, can be altered to change the final properties accordingly.

The process flowsheet, Figure 8.1, describes products with and without cells. Fibres with no cells can be made and stored ready for use, for example, as a cell-free dermal

equivalent. Alternatively, it may be important to seed the fibres with cells, before implantation. Inclusion of cells within an implant can promote the production of growth factors in the wound and thus aid wound healing. The use of autologous cells, although reducing chances of immune reactions, requires extensive cell culture of cells from a biopsy. Patients suffering from severe burns do not have time for this cell culture to occur so they would require either cell free products or one containing allogenic, dermal fibroblasts, which had been screened for transmittable agents. The use of fibronectin fibres on a backing material describes only a dermal equivalent. No epidermal layer is included. These dermal replacements need to be used in conjunction with autografts, for patients with ulcers, where time and donor sites are not limited, whilst a dressing / coating or allograft would be required to cover the dermal replacement for burns patients, to reduce the immediate risk of dehydration. Entire skin substitutes could be produced if a layer of non-immunogenic keratinocytes was seeded on top of the sponge. Increasing the complexity of the product increases the regulations that the product must adhere to, since these combination products lie at the interface of medical device, pharmaceutical and biotechnology regulation.

One of the advantages of using the fibronectin / fibrinogen material is that it is natural. Unfortunately this means that it will be broken down by the body, perhaps in a similar manner to a fibrin clot. To provide directional cues to cells, and ultimately promote the deposition of orientated matrix, the fibronectin may have to be present in the body for weeks or months. Severe dehydration has been described by Yannas (1995) for the stabilisation of collagen materials and results in this thesis also confirmed that dehydration stabilised the material for the duration of a washing step. As an alternative approach, Ahmed *et al.*,(1998a, e) describes the stabilisation of fibronectin materials with micromolar concentrations of copper without compromising cellular adhesion. Copper is released from the fibronectin materials on their disintegration and may act to promote angiogenesis or restore local deficiencies in copper ions, believed to retard wound healing after burn injury (Brown *et al.*,1998b). It is important that these materials will eventually breakdown and it should be considered whether breakdown of the guidance material should occur at the same rate as new matrix deposition (Peters and Mooney, 1997).

This discussion has centred around the production of fibronectin materials and their

physical and cell adhesion properties. These interactions form only a small part of the whole field of tissue engineering and other considerations must be given to biological aspects such as revascularisation of the implant, if such an implant is to succeed. Langer *et al.*, 1995 wrote that cells will not survive if they are > 100 μm from the nearest capillary and so matrix design should allow for the influx of endothelial cells . Peters and Mooney (1997) state that it will take 2.5 hours for an endothelial cell to travel 100 μm in a matrix with pores > 10 μm (Table 1.2) and so matrices must allow such movement. Normally during vascularisation of a fibrin clot, fibronectin promotes the migration of endothelial cells into the area (Colvin, 1989). Brown *et al.* (1998b) describe the breakdown of mats, releasing fibronectin, as an important consideration in the promotion of angiogenesis. These mats, described by Ejim *et al.* (1993) and Brown *et al.* (1994), can be linked with heparin and used as a depot for basic fibroblast growth factor, both of which could have angiogenic properties.

To increase the complexity further, in deep dermal wounds, sweat glands and hair follicles are destroyed. In order for the reconstruction of these, the dermal equivalent would have to contain the appropriate cells for glands and follicles as well as have growth factors incorporated into the materials.

9.1.6. Advantages of fibronectin materials over currently available skin replacement products.

As described in section 1.1.1, there are a variety of commercially available skin replacement technologies. Although some of these products possess some of the properties described below, none possess all.

- derived from a natural, human source
- cell orientation
- can be used with and without pre-seeding with cells. The use of optimised proportions of fibronectin and fibrinogen will promote cell migration from the surrounding tissues
- can be used as a depot for controlled growth factor release
- materials, based on the same manufacturing technology, can be targeted towards different tissue repair applications
- can be stabilised but will also release fibronectin which recruits a variety of cell types,

important in wound healing

This discussion covers the issues considered during the research into a suitable process for large scale production of a fibronectin guidance material. It has highlighted the advantages and disadvantages of each of the materials and raised issues about their development into tissue repair products. Conclusions in response to the project aims are given in section 9.2 and some recommendations for further work in this ever-expanding, complex field are discussed in 9.3.

9.2 Conclusions

9.2.1. Process conclusions

Fibronectin mat making is a useful technique for producing fibronectin guidance scaffolds on a small scale. However, the need for large quantities of purified fibronectin, difficulties in controlling the mat formation process and problems maintaining a continuously clean operating environment do not lend this technique to large scale production.

Purified fibronectin from two sources, glycine supernatant (BPL, Elstree, UK) and cryoprecipitate (PFC, Edinburgh, UK), can be used to form fibronectin mats. A change in the molecule's conformation, to form an open structure, is important for the mat-making process.

PEG fractionation, with 4 and 10 % steps results in precipitates containing 59 - 72 % fibronectin and 28 - 41 % fibrinogen. Further optimisation of this process is required to achieve the appropriate concentration for optimal cell migration.

The effect of changes in environmental conditions, on the conformation of both fibronectin and fibrinogen molecules, have been used to develop two new materials.
Drawn, fibronectin cables, which possess good cell adhesion and alignment properties have been made, however the method of their manufacture was not suitable for large scale production.

Wet spinning has been chosen as a reproducible, reliable and flexible process for the

production of fibronectin guidance materials. Fibres containing both fibronectin and fibrinogen can be wet spun although further optimisation of the process is required to form fibres for specific functions.

Wet spinning is proven technology which can be performed at both large and small scales. It allows for flexibility in altering the feedstock but also requires that the feedstock viscosity is monitored for successful fibre formation.

Altering the feedstock composition using viscosity enhancers, or changing the protein composition will alter final fibre properties.

Solutions with total protein concentration at least 70 mg/mL are required to spin fibres on a small scale. This minimum concentration can be decreased by adding viscosity enhancers. Spinning fibres with diameter < 500 μm requires solutions with an apparent viscosity > 400 mPa s at the shear rate in the capillary or spinneret.

Spinning dopes, with a high protein content, cannot be stored for longer than 2 days without substantial loss in apparent viscosity. Loss of viscosity could be reduced by increasing the final urea concentration in solution and storage at 4°C. Fibre spinning ability was closely related to solution apparent viscosity.

9.2.2. Product conclusions

Drawn fibronectin cables, average diameter 200 μm, have an average dry tensile strength 15 N/mm^2. Twisting the material during formation increases intermolecular interactions and consequently the average tensile strength to 61 N/mm^2. The material's extension at break is low, 3.5-4.9 %, making it brittle and difficult to handle.

The cables are hygroscopic and could be stabilised against dissolution in water by severe dehydration.

Fibronectin and fibrinogen fibres can be spun on a pilot scale, when alginate is used as a viscosity enhancer. The properties of these fibres varied with their composition. Fibres containing a higher protein content have a greater average tensile strength (28 N/mm^2)

than the high alginate fibres (7 N/mm^2) allowing them to be collected and drawn down to a finer diameter using rollers. Dehydration of fibres with acetone results in an increased tensile strength. The high alginate fibres contained numerous voids in the material, caused by trapped air, which lowered their tensile strength. Ridges on the fibre's surface, 1-10 μm diameter are suitable for promoting cell alignment.

The average elongation at break for the wet spun, high protein fibres was 52 % whilst for high alginate fibres it was 21 %. These values describe fibres which are more handleable and easier to process than the fibronectin cables.

From a 1 litre plasma donation, 39 mL of a 4 mg/mL solution for drawing cables can be produced or 1 mL of spinning dope, containing a total 150 mg protein. From these an equivalent volume of cable or fibre can be made although the spun fibres require drawing down to a fine diameter.

Fibronectin cables support the adhesion and alignment of human dermal fibroblasts. Cell orientation occurs due to guidance by micron-diameter fibrils running parallel to the cable's long axis. Using collagen as a backing material, allowed the production of confluent sheets of cells although only cells on top of the fibronectin cables showed orientation with the collagen sponge providing its own directional cues. If the fibronectin cables are placed close enough, ≤ 1 mm apart, the orientation of the cells may be ideal for skin, which requires a network rather than perfect orientation.

The materials show excellent prospects for use in other applications where new tissue orientation is critical, e.g.: nerve, tendon and blood vessels.

9.2.3. Regulatory conclusions

Two virus inactivation steps must be included in the process if pooled plasma is to be used. Solvent / detergent treatment, high concentrations of urea, solution pasteurisation and terminal heat treatment are all methods which could be incorporated into the process, providing that they do not affect the final properties of the material.

GMP guidelines can be applied to the wet spinning process and are already in place for

the production of many potential feedstocks. Detailed documentation is required so that a particular batch of plasma used can be traced to the final product.

A complicated review system for tissue engineered products exists and products will typically be reviewed on a case by case basis.

9.3 Recommendations for future work

9.3.1. Feedstock

Future studies could investigate more controlled PEG fractionation, examining the effects of temperature and pH on the outcome of the fractionation process. The optimum fibronectin / fibrinogen composition must be chosen for cell adhesion / migration and solution viscosity for each application and the composition, for each use, must be controlled within restricted limits under GMP regulations. There is also a need to develop sterile feedstocks with validated virus inactivation steps incorporated into the preparative process. The use of solvent / detergent treated fibronectin / fibrinogen sources from the Factor VIII extraction process should be further examined.

9.3.2. Process

Methods of increasing the protein concentration and viscosity of the spinning dope without the use of viscosity enhancers should be sought. The use of sodium hydroxide as a protein solvent in the formation of spinning dopes is well documented and the use of acetic acid has been described as a stabiliser, for maintaining high viscosity. Protein only feedstocks will not show the same shear thinning properties as those containing alginate. As a consequence these solutions will have different extrusion viscosities and the final properties of the fibre will differ from those described here. The coagulation of high concentration dopes at a pH around the pH of minimum solubility (pH 3-4) should be examined. The high protein concentration may enhance inter-molecular interactions and improve the coagulation above that seen with the low concentration solutions. Acetic acid / sodium chloride combinations have proved popular when animal plasma proteins have been spun. All changes in the process could alter the final product and so the fibre's physical and cell adhesion properties must be tested for each change. The use of other additives such as CMC or dextran should not be overlooked.

The effect of changing the flowrate and the time spent in the coagulation bath should be examined as well as the rate of uptake onto rollers and drawing to an appropriate diameter. A knowledge of the fibre's tensile strength and elongation at break will be required for this.

9.3.3. Scale

Applications other than skin repair, such as tendon and nerve, will require smaller quantities of material than for skin replacement. Therefore small scale production of autologous materials should be investigated as well as the production of large quantities of material for skin replacement products.

9.3.4. Final products

In order to produce realistic replacements, the natural structure and properties of the tissue to be mimicked should be examined. Decisions to be made include, whether a network of fibres are required, such as for skin, or a highly orientated template, e.g.: for nerve and tendon, or a more complex system, e.g. for blood vessels. The pore size, the need to resist compressive forces or provide mechanical strength and elasticity should be considered in scaffold design. The required cell adhesion and migration properties and thus the most appropriate fibronectin / fibrinogen composition must also be considered.

9.3.5. Additional complexities

So far only a simple structure has been described. Consideration for future products could include, the addition of growth factors to enhance repair or induce angiogenesis. Different tissues would require different factors or doses or indeed gradients of doses throughout the tissue. Each factor may bind differently to the fibronectin material and have a varying release profile.

The incorporation of secondary populations of cells for associated functions, other than those essential for repair should be considered, such as those for sweat glands and hair follicles.

Other strategies which could be considered in the future are:

- the use of genetically altered cells, seeded into the implants to produce high levels of the appropriate growth factors, rather than just adding the purified factor.

- the production of recombinant fibronectin and fibrinogen, possessing both cell binding and extracellular matrix binding functions and eliminating virus transmission risks.

10.0. Appendices

Appendix 1. Materials and methods

10.1.1. Assay errors and standard curves

Errors were measured with respect to assay reproducibility.

Calculation of maximum error of the mean

1. Calibration curve for each assay was repeated 10 times, n=10
2. Average value for each concentration was calculated, x
3. Standard deviation for each concentration was calculated, σ
4. Standard error of the mean calculated, where $S.E. = \frac{\sigma}{\sqrt{n}}$
5. 95 % confidence interval calculated, where $C.I. = tS.E.$

t is obtained from t-tables for n-1 degrees of freedom at the 95 % confidence level.

6. Maximum error of mean calculated, where $error = \frac{C.I.}{x} 100$

For each assay the following is shown:

- typical calibration curve
- the maximum error of mean for each dilution on the curve
- range and type of standards used

10.1.1.1. Bradford Protein assay (Bio-Rad)

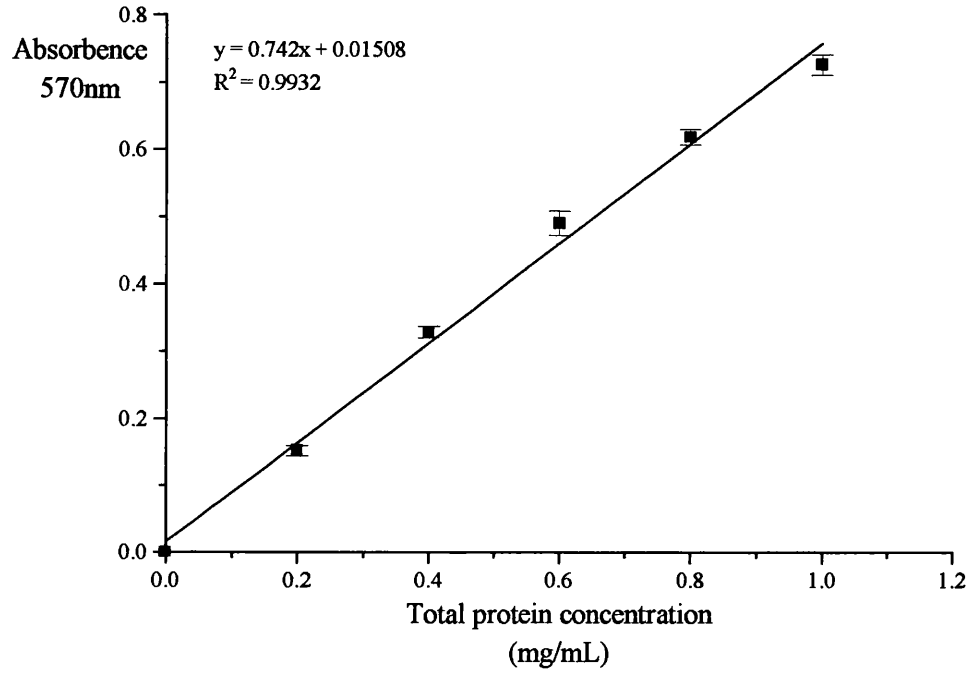


Figure 10.1. *Standard curve for the Bradford protein assay*

Concentration (mg/mL)	0.2	0.4	0.6	0.8	1.0
Maximum error of mean (%)	5.0	2.7	3.7	1.9	2

Standard range: 0.2-1.0 mg/mL

Standards used: Human plasma albumin / globulin mixture (Sigma) diluted with 0.01 M PBS.

Maximum error of mean (95 % confidence interval level): 5 %

Control zero: 0.01 M PBS + dye

10.1.1.2. Fibronectin ELISA

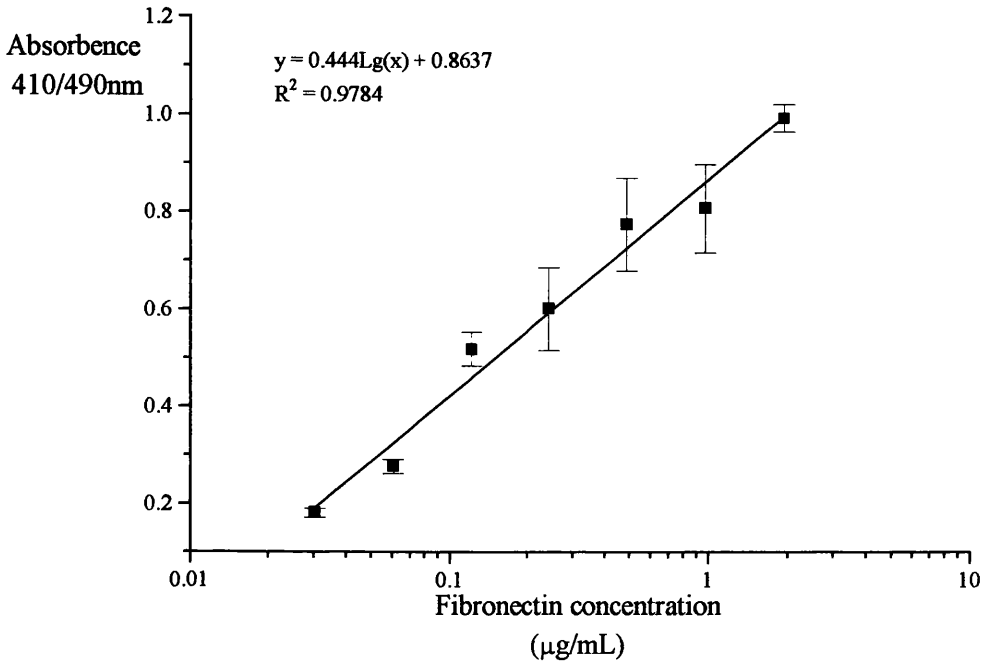


Figure 10.2. *Standard curve for fibronectin ELISA*

Concentration (μg/mL)	1.95	0.97	0.49	0.24	0.12	0.06	0.03
Maximum error of mean (%)	2.8	11.3	12.4	14.2	6.7	5.2	4.7

Standard range: 0.03 - 2 μg/mL

Standards used: affinity chromatography purified fibronectin diluted with 0.01 M PBS

Cross reactivity of assay tested with: albumin and fibrinogen. Antibody controls also included (see section 3.2.1.)

Maximum error of mean (95 % confidence interval level): 14 %

Control zero: 0.01 M PBS plus blocking solution, 1st and 2nd antibodies and pNpp

10.1.1.3. Fibrinogen assay

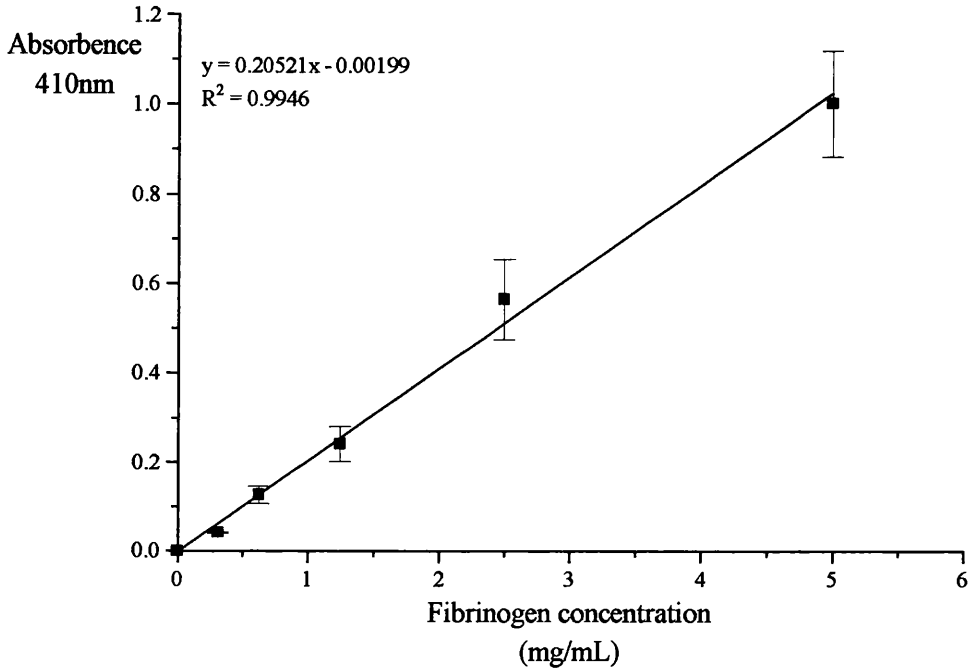


Figure 10.3. *Standard curve for fibrinogen assay*

Concentration (mg/mL)	0.31	0.62	1.25	2.50	5.0
Maximum error of mean (%)	11.8	16.0	16.4	15.5	3.7

Standard range: 0.3-5.0 mg/mL

Standards used: Fibrinogen from fibrin sealant kit (PFC) diluted with 0.01 M PBS.

Assay sensitivity tested with: fibronectin and albumin (see section 3.2.2)

Maximum error of mean (95 % confidence interval level): 16 %

Control zero: 0.01 M PBS plus assay buffer

10.1.1.4. Polyethylene glycol assay

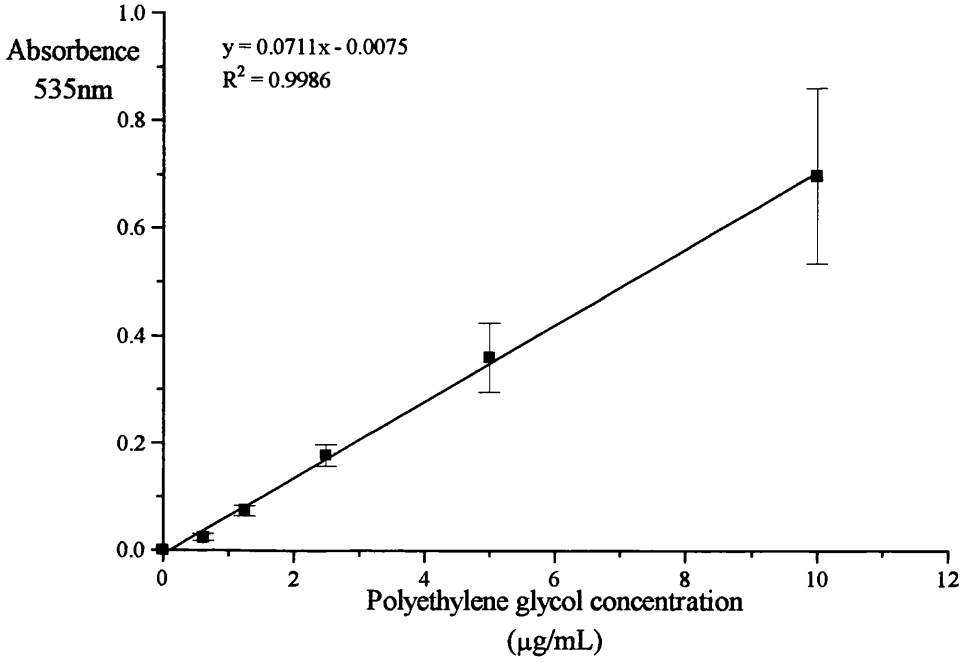


Figure 10.4. *Standard cuve for polyethylene glycol assay*

Concentration (μg/mL)	0.625	1.25	2.5	5.0	10.0
Maximum error of mean (%)	28.5	13.1	11.2	17.8	23.4

Standard range: 0.625-10 μg/mL

Standards used:polyethylene glycol 4000 diluted with 0.01 M PBS

Maximum error of mean (95 % confidence interval level): 29 %

Control zero: 0.01 M PBS plus barium chloride and iodine

10.1.1.5. Albumin assay

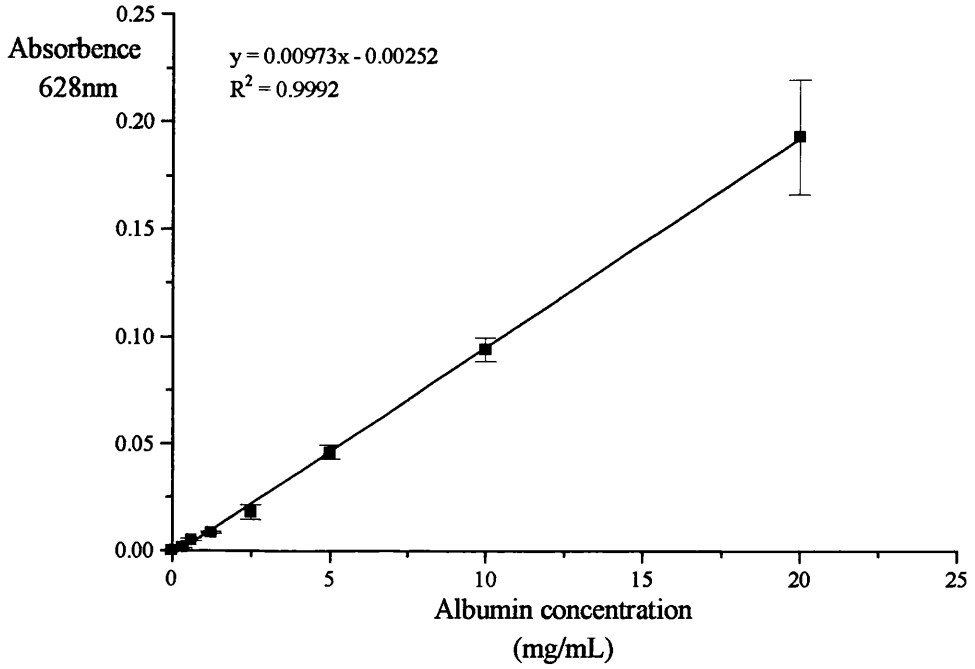


Figure 10.5. *Standard curve for albumin assay*

Concentration (mg/mL)	0.31	0.62	1.25	2.5	5.0	10.0	20.0
Maximum error of mean (%)	10.0	10.5	4.7	19.6	6.9	6.1	13.8

Standard range: 0.3 - 20 mg/mL

Standards used: Bovine serum albumin in 0.01 M PBS

Cross reactivity: Some cross reactivity with fibrinogen / fibronectin

Maximum error of mean (95 % confidence interval level): 20 %

Control zero: 0.01 M PBS plus dye

10.1.2. Electrophoresis buffers and standards

Running buffer

Tris base	2.9 g
Glycine	14.4 g
SDS	1.0 g

Deionized water to 1 litre - final pH 8.3

Sample buffer

0.5 M Tris-HCl, pH 6.8	2.5 mL
10 % (w/v) SDS	4.0 mL
0.1 % bromophenol blue	0.5 mL
Glycerol	2.0 mL
β-mercaptoethanol	0.5 mL
Deionized water to	20.0 mL

Standards (Sigma)

Protein	Molecular weight
Myosin, rabbit muscle	205 000
β-Galactosidase, E.*coli*	116 000
Phosphorylase b, rabbit muscle	97 000
Fructose-6-phosphate kinase, rabbit muscle	84 000
Albumin, bovine serum	66 000
Glutamic dehydrogenase, bovine liver	55 000
Ovalbumin, chicken egg	45 000
Glyceraldehyde-3-phosphate dehydrogenase rabbit muscle	36 000
Carbonic anhydrase, bovine erythrocytes	29 000
Trypsinogen, bovine pancreas	24 000
Trypsin inhibitor, soybean	20 000
α-Lactalbumin, bovine milk	14 200
Aprotinin, bovine lung	6 500

10.1.3. Fixation and dehydration protocol for scanning electron microscopy

PROCEDURE	PURPOSE	TIME
2.5 % glutaraldehyde in 0.1 M phosphate buffer, 4°C	Fixation of proteins	1 hour
0.1 M phosphate buffer	Wash	2 x 5 minutes
1 % osmium tetroxide in 0.1 M phosphate buffer, 23°C	Fixation of phospholipids, fats and polysaccharides	1 hour
0.1 M phosphate buffer	Wash	2 x 5 minutes
Deionized water	Wash	2 minutes
50 % ethanol	Dehydration	15 minutes
70 % ethanol	Dehydration	15 minutes
80 % ethanol	Dehydration	15 minutes
90 % ethanol	Dehydration	15 minutes
100 % ethanol	Dehydration	2 x 30 minutes

All dehydrations and washes occur at 23°C.

Gluteraldehyde, osmium tetroxide (Taab Laboratories Eqpt Ltd, Aldermaston, UK)

Ethanol (BDH)

To make 1 litre 0.1 M phosphate buffer, pH 7.4:

95 mL 0.2 M sodium dihydrogen orthophosphate

405 mL 0.2 M di-sodium hydrogen orthophosphate

500 mL deionized water

Appendix 2. Viscometry

A constant range of shear rates was not used throughout this work. Instead the range of shear rates was chosen to suit the type of solution, its concentration and the measuring device used. When low viscosity solutions were measured, high shear rates were disregarded due to turbulent flow in the rheometer (section 10.2.1) whilst very low torque measurements were also disregarded due to the high error which can occur (section 10.2.2)

10.2.1. Calculation of shear rate at which turbulent flow starts for low viscosity fluids in the concentric cylinders

To calculate the critical Reynolds number, $(N_{Re})_{crit}$, at which transition from laminar to turbulent flow occurs.

$$(N_{Re})_{crit} = 41.3\left(\sqrt{\frac{R_c}{R_c - R_b}}\right) \qquad \text{(equation 10.1)} \quad \text{and}$$

$$(N_{Re})_{crit} = \left\{\frac{\upsilon_b (R_c - R_b)\rho}{\mu}\right\}_{crit} \qquad \text{(equation 10.2)}$$

(Von Wazer *et al.*, 1963)

Calculations based on water as a low viscosity fluid.

R_b	radius of bob	0.0235 m
R_c	radius of cup	0.0240 m
ρ	density of water	998 kg/m^3
μ	viscosity of water	0.001 kg/m s
υ_b	linear velocity of bob	r.p.s*0.14765 m/s

Inserting R_c and R_b into equation 10.1 gives $(N_{Re})_{crit} = 286$

To find the bob linear velocity at the transition point, substitute $(N_{Re})_{crit} = 286$ into equation 10.2

$$286 = \left\{\frac{\upsilon_b(0.0240 - 0.0235)998}{0.001}\right\}$$

$\upsilon_b = 0.57$ m/s

This is equivalent to a bob rotational speed of 3.88 r.p.s.

Figure 10.6 and Table 10.1 show values of bob rotational speed versus shear rate and shear rates and rotational speeds for the concentric cylinders respectively.

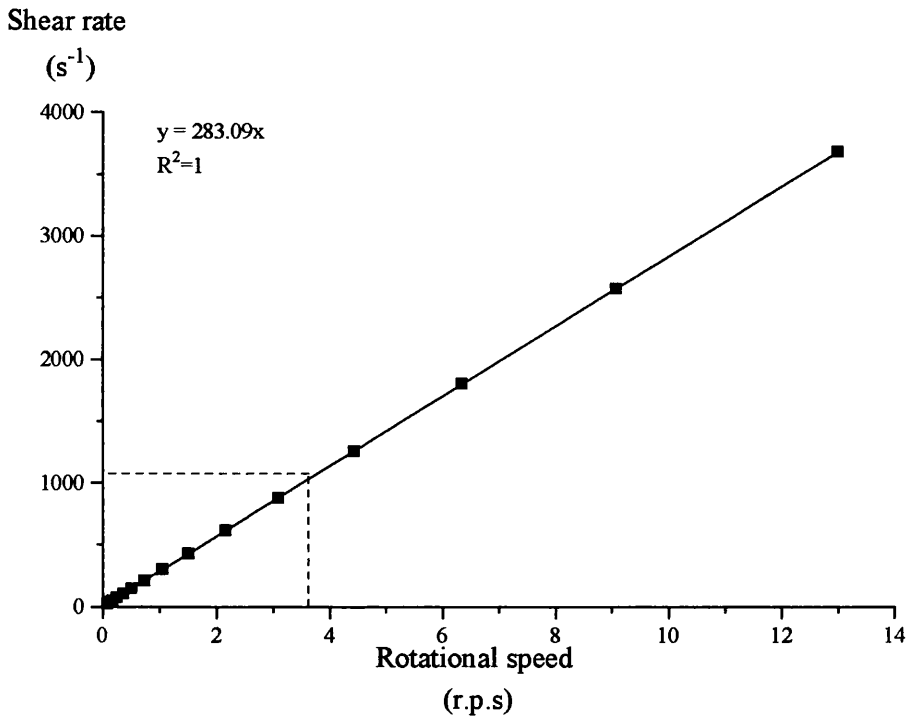


Figure 10.6. *Rotational speed versus shear rate for the concentric cylinder (MS-0) bob.*

Step number	Shear rate (s^{-1})	Rotational speed (r.p.s.)	Bob linear velocity (m/s) υ_b	Re
11	877	3.10	0.46	240
12	1256	4.43	0.65	344
13	1799	6.35	0.94	493
14	2570	9.08	1.34	705
15	3680	13.00	1.92	1009

Table 10.1. *Values of shear rate and rotational speed for the concentric cylinder attachment (MS-0) for the Contraves 115 Rheomat. Bob linear velocities and the corresponding critical Reynolds numbers (Re) have also been calculated.*

From Fig. 10.6, 3.88 r.p.s. generates a shear rate of $1098s^{-1}$. So from Table 10.1. this shear rate occurs between steps 11 and 12 when the concentric cylinders are used to measure viscosity. Thus where the viscosity of low viscosity fluids was measured using the concentric cylinders, values for shear rates including and above $1256s^{-1}$ were discounted because of turbulent flow in the rheometer.

10.2.2. Measurement of power law parameters

The power law relates viscosity to shear rate with the following equation:

$$\mu = K\gamma^{n-1} \qquad \text{(equation 10.3)}$$

where K is the fluid consistency index (mPa s^n) and n is the flow behaviour index (-). For a Newtonian fluid, n=1 and consequently $\mu = K$. For a Newtonian fluid, viscosity is independent of shear rate. Where $n < 1$, the fluid is shear thinning and the viscosity is dependent upon the shear rate. In this case the viscosity is called the apparent viscosity and is quoted with the appropriate shear rate.

The power law also relates shear stress to shear rate:

$$\tau = K\gamma^{n} \qquad \text{(equation 10.4)}$$

Shear stress was plotted against shear rate for the chosen shear rates and curves fitted to determine K and n, as shown in Figure 10.7. As described earlier, measurements made where flow was turbulent, were excluded, as were measurements where a torque reading was less than 1.0 or the torque reading did not increase with shear rate initially, due to the insensitivity of the recording scale.

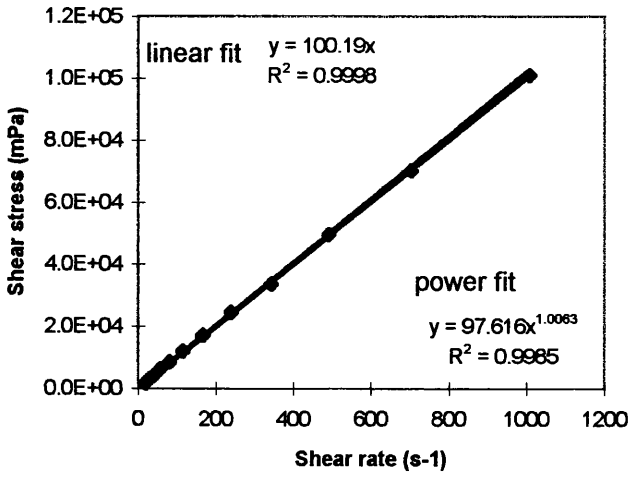


Figure 10.7. *Shear rate versus shear stress for a silicone oil standard. Viscosity was measured using the DIN 125 attachment at 20°C. A power curve was fitted to give the power law parameters K (mPa s^n) and n (-). For this solution K was measured as 97.6 mPa s^n and n as 1.0063. Linear regression, passing through the origin, was used to determine viscosity, measured here as 100.2 mPa s.*

10.2.3. Errors in viscosity measurement

Errors may occur during both measurement of viscosity and interpolation of the produced data. The measuring cups MS-0 (concentric cylinders), DIN 125, 114 and 108 are used to measure solutions with increasing viscosity. The volume of sample required for measurement decreases from 18 mL for the concentric cylinders to 0.75 mL for the DIN 108 attachment.

For some high protein concentration solutions it was not possible to make enough samples for repeat measurements. This was particularly true for samples for the storage experiments (section 6.4.) where only small volumes of sample could be generated. In these instances, measurements were made with a cup and bob which required small volumes of sample but may not accurately measure the appropriate viscosity range. To test the variation in viscosity measurement which could occur because of this problem, silicone oil standards (BDH) with viscosity either 105.3 mPa s or 1090 mPa s and water, all of which are Newtonian fluids, were used to 'calibrate' the rheometer.

Only the concentric cylinders could be used for measuring low viscosity fluids such as water. However they could not be used for measuring the higher viscosity silicone oil

standard. Both DIN 125 and 114 could be used for measuring in the 100 - 1000 mPa s range but DIN 108 could be used to measure the higher viscosity standard only.

Four measurements were made for each standard with each device at 20°C. For each measuring device a value for the % difference between the actual viscosity of the standard and the measured value was calculated (% error from standard) as well as the spread of the measurements (% max. error of mean). Results are given in Table 10.2. In this thesis, if more than one measurement was made then the 95 % confidence interval for the actual data is given. However if only one measurement has been made then the errors calculated here are applicable

Measuring device	Actual fluid (viscosity at 20°C - mPa s)	Shear rate range (s^{-1})	Measured viscosity (mPa s) % error from standard % max. error of mean	Calculated K (mPa s^n) % error from standard % max. error of mean	Calculated n (-) % error from standard % max. error of mean
MS-0	water (1.002)	102-877	0.96 (4.4) (2.9)	0.92 (8.0) (67.0)	1.03 (3.1) (9.6)
MS-0	silicone oil (105.3)	24.3-643	101.4 (3.7) (10.9)	110.3 (4.7) (16.5)	0.99 (1.4) (1.1)
DIN 125	silicone oil (105.3)	27.9-1007.2	100.9 (4.2) (0.6)	114.0 (8.3) (9.1)	0.98 (1.9) (1.4)
DIN 125	silicone oil (1090)	6.65-1007.2	1003 (7.9) (0.5)	1112 (2.0) (9.0)	0.96 (1.5) (1.5)
DIN 114	silicone oil (105.3)	167.6-1007.2	108.2 (2.8) (4.5)	340.4 (223.2) (45.9)	0.84 (16.2) (7.6)
DIN 114	silicone oil (1090)	19.5-1007.2	1112 (2.0) (14.8)	1230 (12.8) (29.2)	0.99 (1.2) (2.7)
DIN 108	silicone oil (1090)	81.8-1007.2	1094 (0.4) (5.7)	2099 (92.6) (34.2)	0.91 (10.2) (4.2)

Table 10.2. *Summary of error measurements for each measuring device.*

An estimated error for each device

The percentage error from standard and the max. error of the mean have been considered to give an overall error for each device. This is considered an estimate, because the accuracy of the device is dependent on the viscosity of the solution.

Concentric cylinders (MS-0)	viscosity ± 14 %	K ± 70 %	n ± 12 %
DIN 125	viscosity ± 8 %	K ± 18 %	n ± 3 %
DIN 114	viscosity ± 17 %	K ± 372 %	n ± 22 %
DIN 108	viscosity ± 6 %	K ± 158 %	n ± 22 %

The range of viscosity and n values corresponds to ± no greater than 20 % in most cases. However the K values appear to fluctuate widely and their values should be used to evaluate trends rather than actual values.

Solutions of 100 % glycerol were used to confirm the calibrations. Results are shown in Figure 10.8

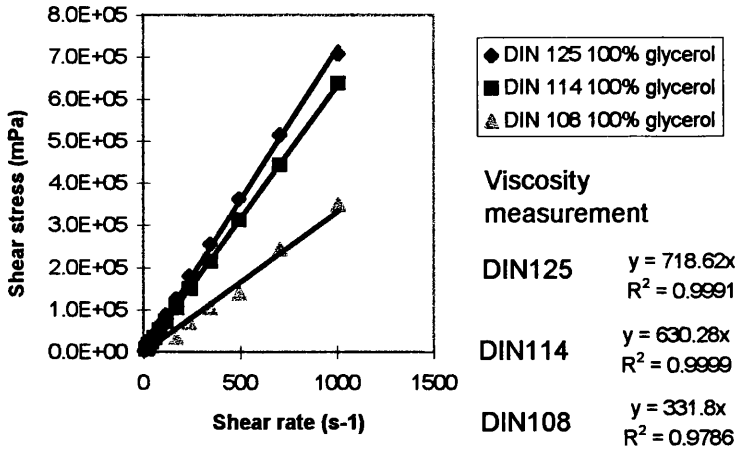


Figure 10.8. *Shear rate versus shear stress for 100 % glycerol. Measurements for all three cup and bobs were made at 23°C. A linear fit was used to determine the viscosity of the solution, 719, 630 and 332 mPa s for DIN 125, 114 and 108 respectively. Fitting a power curve gave K values of 688, 454 and 69 mPa s^n whilst values for n were 1.01, 1.06 and 1.24 for 125, 114 and 108 attachments.*

The published literature value for the viscosity of 100 % is 750 mPa s. The viscosity measured by DIN 114 is 12 % lower than that measured using DIN 125 and is within the level of accuracy quoted above. However the value from DIN 108 is 54 % lower than

that using DIN125 and 47 % less than that with DIN 114. This increased error is caused by the viscosity of the solution being outside the measurable range for this bob.

Appendix 3 Preparation of high concentration protein solutions

10.3.1. Addition of PEG precipitate to urea to form spinning dopes - calculated versus measured protein concentrations

High concentration protein solutions were required for fibre spinning. However, it was difficult to measure the concentration of protein in solution accurately for concentrations greater than 70 mg/mL or in the presence of viscosity enhancers, using the Bradford protein assay, as the high viscosity of the solution made pippetting unreliable. Instead the amount of protein added to solution was calculated from the known composition of the precipitate, as described below:

Assume 1 g of freshly thawed precipitate is added to 1 mL urea
The precipitate contains 25 % solid material and 75 % phosphate buffer (dry weight measurement)
The solid material contains both PEG and protein in the ratio 0.6:1.0 (w/w)
Thus in 1 g of precipitate there is 0.75 mL phosphate buffer
0.16 g protein
0.09 g PEG
Total volume of protein in solution is now 1.75 mL

so protein concentration = $\left(\frac{0.16}{1.75}\right) * 1000 = 91 mg / mL$

Calculated concentrations are compared to measured concentrations in Figure 10.9.

For the pilot scale spinning and storage experiments, chapter 6.0, the protein concentrations were calculated, using the above method, instead of measured.

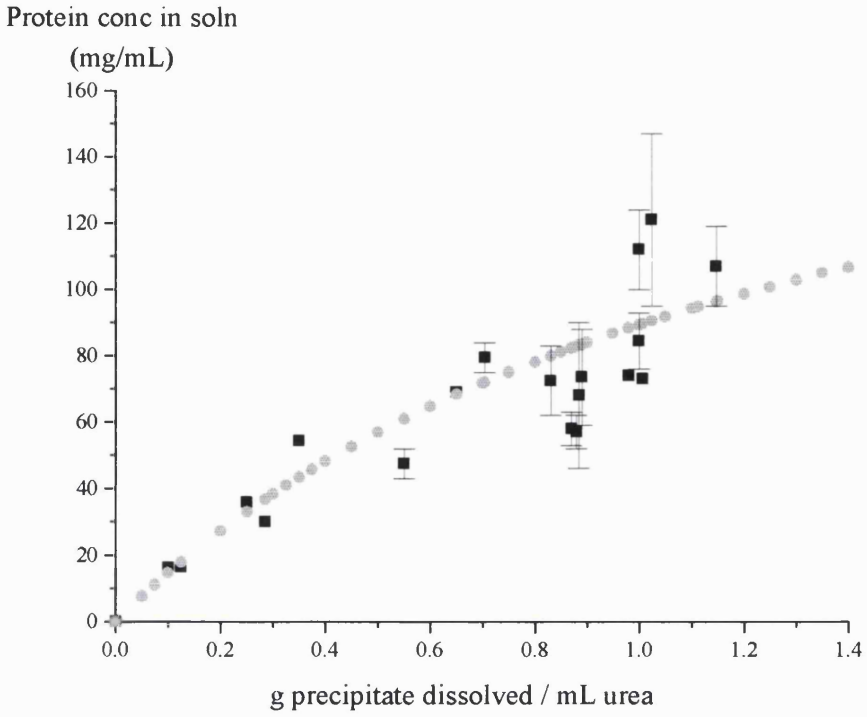


Figure 10.9. *Protein concentrations measured using the Bradford protein assay,* ■ *and concentrations calculated from precipitate composition,* ●. *Error bars represent the range of concentrations measured in repeated samples using the protein assay. Values are lower than expected when 0.8-1.0 g precipitate were dissolved / mL urea suggesting that insufficient solution was drawn into the pippette for the assay. Values higher than the calculated concentrations were believed to be due to protein precipitation in the dilution buffer (1.0-1.2 g precipitate / mL urea).*

References

Abercrombie, M.: The crawling movement of metazoan cells. In: Cell Behaviour (Ed. Bellairs, R.; Curtis, A.; Dunn, G.) Publd. Cambridge University Press, Cambridge, (1982), pp. 19-43

Agren, M. S.: Four alginate dressings in the treatment of partial thickness wounds: a comparative experimental study. British Journal of Plastic Surgery (1996) 49 129-134

Ahmed, Z.; Idowu, B.D.; Brown, R.A.: Stabilisation of fibronectin mats with micromolar concentrations of copper. Biomaterials (1998a) In press.

Ahmed, Z.; Underwood, S.; Brown, R.A.: Production of large fibres of fibronectin as potential conduits for repair of long peripheral nerve lesions. In preparation. (1998b)

Ahmed, Z.; Underwood, S.; Brown, R.A.: High concentrations of fibrinogen inhibit cell adhesion and migration on fibronectin / fibrinogen composite strands. In preparation (1998c)

Ahmed, Z.; Underwood, S.; Brown, R.A.: Adhesion and migration of Schwann cells on fibronectin / fibrinogen strands. In preparation (1998d)

Ahmed, Z.; Underwood, S.; Brown, R.A.: Copper and zinc ions stabilise large fibres. In preparation (1998e)

Alexander, S.S.; Colonna, G.; Edelhoch, H.: The structure and stability of human cold-insoluble globulin. J.B.C. (1979) 254 (5) 1501-1505

Anderson, J.P.; Cappello, J.; Martin, D.C.: Morphology and primary crystal structure of a silk-like protein polymer synthesized by genetically engineered *Escherica coli* bacteria. Biopolymers (1994) 34 1049-1057

Askman, L.; Wetzel, P.; Isely, A.: Process for spinning fish proteins. U.S. Patent 4340612 (1982)

Asteury, W. T.: Artificial protein fibres: their conception and preparation. Nature (1945) 155 501-503

Atherton, B.T.; Hynes, R.O.: A difference between plasma and cellular fibronectins located with monoclonal antibodies. Cell (1981) 25, 133-141

Balmaceda, E.; Rha, C.: Spinning of zein. J. Fd. Sci. (1974) 39 226-229

Barrera, D.A.; Zylstra, E.; Lansbury, P.T.; Langer, R.: Synthesis and RGD peptide modification of a new biodegradable copolymer: poly (lactic acid-co-lysine). J.Am.Chem.Soc. (1993) 115 11010-11011

Bell, D. J.: The physical characteristics of protein precipitates and their centrifugal recovery. PhD. thesis , University of London (1982).

Bell, D. J.; Hoare, M.; Dunnill, P.: The formation of protein precipitates and their

centrifugal recovery. Advances in Biochemical Engineering / Biotechnology (1983) 26 1-70

Bell, E.: Strategy for the selection of scaffolds for tissue engineering. Tissue Engineering (1995) 1 (2) 163-170

Bell, E.; Ehrlich, H. P.; Buttle, D. J.; Nakatsuji, T.: Living tissue formed in vitro and accepted as skin-equivalent tissue of full thickness. Science (1981) 211 1052-1054

Ben-Hur, E.; Horowitz, B.: Virus inactivation in blood. AIDS (1996) 10 (11) 1183-1190

Benecky, M.J.; Wine, R.W.; Kolvenbach, C.G.; Mosesson, M.W.: Ionic strength and pH-dependent conformational states of human plasma fibronectin. Biochemistry (1991) 30 4298-4306

Berger, S.A.; Goldsmith, W.; Lewis, E.R. (Eds.) In: Introduction to Bioengineering. Publd. Oxford University Press, Oxford. (1996) pp.342-343

Berthod, F., Hayek, D., Damour, O., Collombel, C.: Collagen synthesis by fibroblasts cultured within a collagen sponge. Biomaterials (1993) 14 (10) 749-754

Berthod, F.; Sahuc, F.; Hayek, D.; Damour, O.; Collombel, C.: Deposition of collagen fibril bundles by long-term culture of fibroblasts in a collagen sponge. J. Biomed. Mater. Res. (1996) 32 87-94

Berthold, W.; Walter, J.: Protein purification: aspects of processes for pharmaceutical products. Biologicals (1994) 22 135-150

Binder, B.; Nemeth, P.: Fibronectin solution suitable for use in humans and process for its preparation. U.S. Patent 4727059 (1988)

Booth, J.E. (Ed.) In: Principles of textile testing - an introduction to physical methods of testing textile fibres, yarns and fabrics. Publd. Butterworths (1983)

Boughton, B.J.; Simpson, A.W.: The biochemical and functional heterogeneity of circulating human plasma fibronectin. Biochem. Biophys. Res. Comm. (1984) 119 (3), 1174-1180

Bouhadir, K.H.; Yue, I.; Hausman, D.; Mooney, D.: Cross-linked limit-oxidized alginate for tissue engineering applications. J. Dent. Res. 77 (AADR Abstracts) (1998) 1169

Boyce, S.T.: Cultured skin substitues: a review. Tissue Engineering (1996) 2 (4) 255-266

Bramaud, C.; Aimar, P.; Daufin, G.: Whey protein fractionation: isoelectric precipitation of α-lactalbumin under gentle heat treatment. Biotech. Bioeng. (1997) 56 (4) 390-397

Brower, V.: The FDA "reinvents" human cell and tissue regulations. Nature Biotechnology (1997) 15 (5) p.411.

Brown, E.J.; Bohnsack, J.F.; O'Shea, J.J.; McGarr, J.: Evidence for a conformational change in human fibronectin which occurs between 4 and 37°C. Mol. Immunol. (1987) 24 (3) 221-230

Brown, R.A.; McFarland, C.D.: Therapeutic uses of cell-matrix adhesive proteins. Current Opinion in Therapeutic Patents (1993) 1117-1140

Brown, R. A.; Blunn, G. W.; Ejim, O. S.: Preparation of orientated fibrous mats from fibronectin: composition and stability. Biomaterials (1994) 15 (6) 457-464

Brown, R. A.; Porter, R. A.; Ejim, O.S.; Idowu, B.D.; Blunn, G.W.: Control of human dermal fibroblast collagen orientation by an artificial fibrillar fibronectin scaffolding. In preparation (1998a)

Brown, R. A.; Terenghi, G.; McFarland, C. D.: Contact guided angiogenesis and tissue engineering. In: The New Angiotherapy. (Ed.: Fan, T.). Publd. The Humanic Press, Tstowa, New Jersey. In press (1998b)

Burlington, D.B.: FDA's approach to international harmonization of biomaterials and tissue-engineered products. Tissue Engineering (1997) 3(1) 79-83

Cappello, J.; Crissman, J.W.: The design and production of bioactive protein polymers for biomedical applications. Abstracts of Papers of ACS (1990) 199 Meeting pt 2 poly 66

Carr, M. E.; Gabriel, D.A.; McDonagh, J.: Influence of factor XIII and fibronectin on fiber size and density in thrombin-induced fibrin gels. J. Lab. Clin. Med. (1987) 110 (6) 747-752

Cavallaro, J. F.; Kemp, P. D.; Kraus, K. H.: Collagen fabrics as biomaterials. Biotech. Bioeng. (1994) 43 (8) 781-791

Chiquet, M.; Eppenberger, H.M.; Turner, D.C.: Muscle morphogenesis: evidence for an organizing function of exogenous fibronectin. Developmental Biology (1981) 88 220-235

Chou, L.; Firth, J.D.; Uitto, V.; Brunette, D. M.: Substratum surface topography alters cell shape and regulates fibronectin mRNA level, mRNA stability, secretion and assembly in human fibroblasts. J. Cell Sci. (1995) 108 1563-1573

Clark, P.; Connolly, P.; Moores, G.R.: Cell guidance by micropatterned adhesiveness *in vitro*. J.Cell Sci. (1992) 103 287-292

Clark, R.A.F.: Potential roles of fibronectin in cutaneous wound repair. Arch. Dermatol. (1988) 124 201-206

Colvin, R.B.: Fibronectin in wound healing. In: Fibronectin (Ed. Mosher, D.F.) Publd. Academic Press Inc, London (1989) pp 213-250

Cooper, M.L.; Spielvogel, R.L.: Artificial skin for wound healing. Clinics in Dermatology (1994) 12 183-191

Corbett, S.A.; Wilson, C.L.; Schwarzbauer, J.E.: Changes in cell spreading and cytoskeletal organization are induced by adhesion to a fibronectin - fibrin matrix. Blood (1996) 88 (1) 158-166

d'Hemecourt, P.A.; Smiell, J.M.; Hugill, J.V.; Prigoff, M.M.: The effect of topically

applied Sodium Carboxymethylcellulose gel in non-healing, lower extremity ulcers in patients with diabetes. Abstract - 7th Annual Meeting of European Tissue Repair Society, Koln, Germany, August 23-26 (1997)

den Braber, E.T.; de Ruijter, J.E.; Ginsel, L.A.; von Recum, A.F.; Jansen, J.A.: Quantitative analysis of fibroblast morphology on microgrooved surfaces with various groove and ridge dimensions. Biomaterials (1996) 17 2037-2044

Doillon, C. J.; Silver, F. H.: Collagen-based wound dressing: effects of hyaluronic acid and fibronectin on wound healing. Biomaterials (1986) 7 3-8

Doillon, C.J.; Silver, F. H.; Berg, R. A.: Fibroblast growth on a porous collagen sponge containing hyaluronic acid and fibronectin. Biomaterials (1987) 8 195-200

Doillon, C. J.; DeBlois, C.; Cote, M.; Fournier, N.: Bioactive collagen sponge as connective tissue substitute. Mat. Sci and Eng. (1994) C2 43-49

Donaldson, D.J.; Mahan, J.T.: Fibrinogen and fibronectin as substrates for epidermal cell migration during wound closure. J. Cell Sci. (1983) 62 117-127

Doolittle, R.F.: Fibrinogen and fibrin. In: The Plasma Proteins, structure, function and genetic control, II (Ed. Putnam, F.W.), Academic Press Inc., London (1975) pp.110-161

Dormont, D.; Deslys, J.P.; Boussin, F.; Vaslin, B.; Gras, G.; Vogt, G.; Dubeaux, D.; Court, L.: Inactivation of unconventional slow viruses and HIV in human extractive biological products used in human therapy.In: Biotechnologie des proteines du plasma (Biotechnology of plasma proteins). Eds (Stoltz, J.F.; Rivat, C.) Colloque INSEREM 175 (1989) pp. 355-364

Doyle, J.W.; Roth, T.P.; Smith, R.M.; Li, Y.; Dunn, R.M.: Effect of calcium alginate on cellular wound healing processes modeled *in vitro* J.Biomed. Mater. Res. (1996) 32 561-568

Drumheller, P.D.; Hubbell, J.A.: Polymer networks with grafted cell adhesion peptides for highly biospecific cell adhesive substrates. Anal. Biochem. (1994) 222 380-388

Dunn, G.A.: Contact guidance of cultured tissue cells: a survey of potentially relevant properties of the substraturm. In: Cell Behaviour (Ed. Bellairs, R.; Curtis, A.; Dunn, G.) Publd. Cambridge University Press, Cambridge, (1982), pp. 247-276

Dunn, G.A.; Heath, J.P.: A new hypothesis of contact guidance in tissue cells. Exp. Cell Res. (1976) 101 1-14

Dunn, G.A.; Ebendal, T.: Contact guidance on orientated collagen gels. Exp. Cell Res. (1978) 111 475-479

Durfor, C.N.: Biotechnology Biomaterials: A global regulatory perspective for tissue-engineered products: summary report and future directions. Tissue Engineering (1997) 3(1) 115-120

Eaglstein, W. H.; Falanga, V.: Tissue engineering and the development of Apligraf®, a human skin equivalent. Clinical Therapeutics (1997) 19 (5) 895-905

East, G. C.; McIntyre, E.; Qin, Y.: The production of fibres from chitosan. In: Chitin and Chitosan. (Eds. Skjak-Braek, G.; Anthonsen, T.; Sandford, P.) Publd. Elsevier, London (1989). pp.757-763

Eggerman, T.L.; Patterson, A.P.: The United States Food and Drug Administration paradigm for the regulation of tissue-engineered products. Tissue Engineering (1997) 3(1) 109-113

Ejim, O.S.; Blunn, G.W.; Brown, R.A.: Production of artificial-orientated mats and strands from plasma fibronectin: a morphological study. Biomaterials (1993) 14 (10) 743-748

Englard, S.; Seifter, S.: Precipitation techniques. In: Guide to protein purification. Methods in Enzymology 182 (Ed. Deutscher, M.P.) Publd. Academic Press, Inc, London, (1990) pp.285-300

Engvall, E.; Ruoslahti, E.: Binding of a soluble form of fibroblast surface protein, fibronectin to collagen. International Journal of Cancer (1977) 20 1-5

Esouf, M. P.: The thrombin - fibrinogen reaction and the stabilisation of fibrin. In: Human Blood Coagulation, Haemostasis and Thrombosis. (Ed. Biggs, R.) Publd. Blackwell Scientific Publ., Oxford (1972). pp. 32-41

Exner, T.; Burridge, J.; Power, P.; Rickard, K.A: An evaluation of currently available methods for plasma fibrinogen. American Journal of Clinical Pathology (1979) 71 521-7

Farrugia, A.; Grasso, S.; Douglas, S.; Harrap, R.; Oates, A.; Young, I.; James, J.; Whyte, G.: Modulation of fibrinogen content in cryoprecipitate by temperature manipulation during plasma processing. Transfusion (1992) 32 (8) 755-759

FDA (The Food and Drug Administration). Proposed approach to regulation of cellular and tissue-based products. J. Hematotherapy (1997) 6 195-212

Ferry, J.D.: Protein Gels. Advances in Protein Biochemistry (1948) 4 1-78

Fogerty, F.J.; Mosher, D.F.: Mechanisms for organization of fibronectin matrix. Cell Differentiation and Development (1990) 32 439-450

Foster, P.R.: Assessment of the potential of plasma fractionation processes to remove causative agents of Creutzfeldt-Jakob diease (CJD). Poster abstract: Scotblood'98, University of Stirling, 5-6 June (1998)

Foster, P.R.; Cuthbertson, B.: Procedures for the prevention of virus transmission by blood products. In: Blood, Blood Products and HIV. (Eds. Madhok, R.; Forbes, C.D.; Evatt, B.L.) Publd. Chapman and Hall Medical, London (1994) pp. 207-248

Freed, L. E.; Vunjak-Novakovic, G.; Biron, R. J.; Eagles, D. B.; Lesnoy, D.C.; Barlow, S. K.; Langer, R.: Biodegradable polymer scaffolds for tissue engineering. Bio/Technology (1994) 12 689-693

Freeman, M.F.: The European regulatory scene as applied to tissue-engineered products. Tissue Engineering (1997) 3(1) 91-94

Gallico, G.G.; O'Connor, N.E.: Engineering a skin replacement. Tissue Engineering (1995) 1 (3) 231-240

Gere, J.M.; Timoschenko, S.P. (Eds): Mechanics of materials 3rd edition. Publd. Chapman and Hall, London (1991) pp. 9-18

Glaser, V.: U.S. skin-replacement market could heat up. Bio/Technology (1995) 13 933

Gomez-Lechon, M.J., Castell, J.V.: Enzyme-linked immunosorbent assay to quantify fibronectin. Analytical Biochemistry (1985) 145 1-8

Gomez-Lechon, M.J., Castell, J.V.: Measurement of fibronectin in human body fluids. J. Clin. Chem. Clin. Biochem. (1986) 24 333-339

Greiling, D; Clark, R.A.: Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix. J. Cell Sci. (1997) 110 861-870

Grinnell, F.: Fibronectin and wound healing. J. Cell. Biochem. (1984) 26 107-116

Grinnell, F.: Fibroblasts, myofibroblasts, and wound contraction. J. Cell Biol. (1994) 124 (4) 401-404

Grinnell, F.; Feld, M.K.: Initial adhesion of human fibroblasts in serum-free medium: possible role of secreted fibronectin. Cell (1979) 17 117-129

Grinnell, F.; Feld, M.; Minter, D.: Fibroblast adhesion to fibrinogen and fibrin substrata: requirement for cold-insoluble globulin (plasma fibronectin). Cell (1980) 19 517-525

Grinnell, F.; Fukamizu, H.; Pawelek, P.; Nakagawa, S.: Collagen processing, crosslinking, and fibril bundle assembly in matrix produced by fibroblasts in long-term cultures supplemented with ascorbic acid. Exp. Cell Res. (1989) 181 483-491

Hansbrough, J.F.: What criteria should be used for designing artificial skin replacements and how well do the current grafting materilas meet these criteria?. The J. of Trauma (1984) 24 (9) S31-35

Hansbrough, J. F.; Morgan, J.; Greenleaf, G.; Underwood, J.: Development of a temporary living skin replacement composed of human neonatal fibroblasts cultured in Biobrane, a synthetic dressing material. Surgery (1994) 115 633-644

Hao, Y.L.; Ingham, K.C.; Wickerhauser, M.: Fractional precipitaion of proteins with polyethylene glycol. In: Methods in plasma protein precipitation. (Ed. Curling, J. M.) Publd. Academic Press, London, (1980) pp. 57-74

Harding, S.I.: Alignment of human dermal fibroblasts by fibronectin cables. MSc. thesis, University of London (1996)

Harding, S.I.; Underwood, S.; Brown, R.A.; Dunnill, P.: Assessment of cell alignment by fibronectin multi-fibre cables capable of large scale production. In preparation (1998)

Hellman, K.; Biotechnology Biomaterials: a global regulatory perspective for tissue-engineered products: a preface. Tissue Engineering (1997) 3(1) 77-78

Hiemstra, H.; Tersmette, M.; Vos, A.H.V.; Over, J.; van Berkel, M.P.; de Bree, H.: Inactivation of human immunodeficiency virus by gamma radiation and its effect on plasma and coagulation factors. Transfusion (1991) 31 (1) 32-39

Homandberg, G. A.: Characterization of the interactions of an amino-terminal fibronectin fragment with the native molecule: implications for polymerization of fibronectin. Biopolymers (1987) 26 2087-2098

Horowitz, B.:Investigations into the application of tri(n-butyl) phosphate / detergent mixtures to blood derivatives. In: Virus inactivation in plasma products (Ed. Morgenthaler, J.J.) Publd. Karger, Basel, Switzerland (1989) pp.83-96

Horowitz, B.; Lippin, A.; Chang, R. W.; Shulman, J.; Vandersande, J.; Styker, M.H.; Woods, K. R.: Preparation of antihemophilic factor and fibronectin from human plasma cryoprecipitate. Transfusion (1984) 24 (4) 357-362

Horowitz, B.; Chang, M. Y.: Preparation of fibronectin for therapeutic administration. In: Fibronectin (Ed. Mosher, D. F.). Publd. Academic Press Inc., London (1989) pp. 441-455

Huang, F.; Rha, C.K.: Rheological properties of single-cell protein concentrate: dope formation and its flow behaviour. J.Fd. Sci. (1971) 36 1131-1134

Huang, F. F.; Rha, C.: Protein structures and protein fibers - a review. Polymer Eng. Sci. (1974) 14 (2) 81-91

Huang, H.C.; Hammond, E.G.; Reitmeier, C.A.; Myers, D.J.: Properties of fibers produced from soy protein isolate by extrusion and wet spinning. J.A.O.C.S. (1995) 72 (12) 1453-1460

Hubbell, J.A.; Langer, R.: Tissue Engineering. C&EN (1995) March 13 42-54

Hynes, R.: Molecular biology of fibronectin. Ann. Rev. Cell Biol. (1985) 1 67-90

Hynes, R. O.: (Ed.) In: Fibronectins. Publd. Springer-Verlag, (1990)

Ingham, K.C.: Precipitation of proteins with polyethylene glycol. In: Guide to protein purification. Methods in Enzymology 182 (Ed. Deutscher, M.P.) Publd. Academic Press, Inc, London, (1990) pp.301-306

Iuliano, D.J.; Saavedra, S.S.; Truskey, G.A.: Effect of the conformation and orientation of adsorbed fibronectin on endothelial cell spreading and strength of adhesion. J. Biomed. Mater. Res. (1993) 27 1103-1113

Johnson Langer, L.: Conference spotlights Tissue Engineering. Genetic Engineering News. (1995) Jan 1, pp. 1, 44-47

Johnson Langer, L.: Pivotal trial of artificial skin advances tissue engineering industry. Genetic Engineering News (1996) Jan 15, p.25

Kaplan, A., Szabo, L.L.(Eds.): In: Clinical Chemistry: Interpretation and techniques. Publd. Lea & Febinger, Philadelphia. (1983), pp.159-161

Kaplan, J.E; Snedeker, P.W.: Maintenance of fibrin solubility by plasma fibronectin. J. Lab. Clin. Med. (1980) 96 (6) 1054-1061

Kerner, B.: Good manufacturing practice and viral safety. Blood Coagulation and Fibrinolysis (1995) 6 (2) S15-S19

Khan, M.Y.; Medow, M.S.; Newman, S.A.: Unfolding transitions of fibronectin and its domains - stabilization and structural alteration of the N-terminal domain by heparin. Biochem. J. (1990) 270 33-38

Khan, M.Y.; Newman, S.A.: The salting out behaviour of human plasma fibronectin and its possible correlation with heparin-induced cryoprecipitation of the protein. Biochem. International (1991) 23(1) 1-7

Kimizuka, F.; Ohdate, Y.; Kawase, Y.; Shimojo, T.; Taguchi, Y.; Hashino, K.; Goto, S.; Hashi, H.; Kato, I.; Seikiguchi, K.; Koiti Titani: Role of type III homology repeats in cell adhesive function within the cell-binding domain of fibronectin. J.B.C. (1991) 266 (5) 3045-3051

Kistler, P.; Friedl, H.: Ethanol precipitation. In: Methods of plasma protein fractionation. (Ed. Curling, J. M.) Publd. Academic Press, London (1980) pp. 3-15

Kleinman, H.K.; Luckenbill-Edds, L.; Cannon, F.W.; Sephel, G.C.: Use of extracellular matrix components for cell culture. Anal. Biochem. (1987) 166 1-13

Langer, R.; Vacanti, J.P.: Tissue engineering. Science (1993) 260 920-926

Langer, R.; Vacanti, J.P.; Vacanti, C.A.; Atala, A.; Freed,. L.E.; Vunjak-Novakovic, G.: Tissue engineering: biomedical applications. Tissue Engineering. (1995) 1 (2) 151-161

Launay, B.; Lisch, J.M.: Rheological properties of sunflower and faba bean protein dopes. In: Food texture and rheology. (Ed. Sherman, P.) Publd. Academic Press, London (1979) pp.223-244

Lee, C.; Rha, C.K.:Rheological properties of proteins in solution. In: Food texture and rheology. (Ed. Sherman, P.) Publd. Academic Press, London (1979) pp.245-263

Lee, J.H.; Kim, W.G.; Kim, S.S.; Lee, H.B.: Development and characterization of an alginate-impregnated polyester vascular graft. J.Biomed. Mater. Res. (1997) 36 209-215

Lerner, A. B.; Greenberg, G.R.: A homomolecular serum protein with anomalous solubilities. J.B.C. (1946) 162 429-439

Lillford, P.J.; Wright, D.J.: Influence of isoelectric precipitation on the solubility of soya bean protein. J.Sci. Food. Agric. (1981) 32 (4) 315-327

Lillie, J. H.; MacCallum, D. K.; Jepsen, A.: The structure of subcultivated stratified squamous epithelium grown on collagen rafts. Exp. Cell Res. (1980) 125 153-165

Lundgren, H.P.: Synthetic fibers made from proteins. Advances in Protein Chemistry (1949) 5 305-351

Mabbot, N.A.:Symposium talk. Abstract: Mabbut, N.A.; Brown, K.L.; Farquhar, C.F.;

Bruce, M.E.: PrP and the immune system. Scotblood'98, University of Stirling, 5-6 June (1998)

MacGregor, I.; Drummond, O.; Hornsey, V.; MacGregor, M.; MacDonald, S.; Pepper, D.; Prowse, C.; Barclay, R.; Bessos, H.; Turner, M.; Foster, P.; Kirby, L.; Birkett, C.; Barnard, G.; Hope, J. Application of a dissociation enhanced lanthanide fluoroimmunoassay (Delfia®) for the cellular form of prion protein. Poster abstract: Scotblood'98, University of Stirling, 5-6 June (1998)

Mackie, I. M.; Thomson, B. W.: The preparation and assessment of spun fibres from fish proteins using a wet spinning process. J. Fd. Technol (1982) 17 483-498

MacLeod, A.J.; Dickson, I.H.; Foster, P.R.: Pasteurization of coagulation factor concentrates. Thromb. Haemost (1983) 50 432

Markovic, Z.; Engel, J.; Richter, H.; Hormann, H.: Descrimination of different domains in fibronectin on the basis of their stability against urea denaturation. Hoppe Seyler's Physiol. Z. (1983) 364 551-561

Markovic, Z.; Lustig, A.; Engel, J.; Richter, H.; Hormann, H.: Shape and stability of fibronectin in solutions of different pH and ionic strength. Hoppe-Seyler's Z. Physiol. Chem. (1983b) 364 1795-1804

Martin, P.: Wound healing - aiming for perfect skin regeneration. Science (1997) 276 75- 81

Maruguchi, T.; Maruguchi, Y.; Suzuki, S.; Matsuda, K.; Toda, K.; Isshiki, N.: A new skin equivalent: keratinocytes proliferated and differentiated on collagen sponge containing fibroblasts. Plastic and Reconstructive Surgery (1994) 93 (3) 537-544

Matsuda, K.; Suzuki, S.; Isshiki, N.; Ikada, Y.: Re-freeze dried bilayer artificial skin. Biomaterials (1993) 14 (13) 1030-1035

MCA (Medicines Control Agency). Rules and Guidance for Pharmaceutical Manufacturers. Publd. HMSO (1997)

McIntosh, R.V.; Griffin, B.; Foster, P.R.: The margin of safety from transmission of blood borne agents in a virus inactivated factor VIII concentrate (Liberate® HT). Poster abstract: Scotblood'98, University of Stirling, 5-6 June (1998)

McKeown-Longo, P.J.; Mosher, D.F.: The assembly of the fibronectin matrix in cultured human fibroblast cells. In: Fibronectin (Ed. Mosher, D.F.) Publd. Academic Press Inc., London (1989) pp. 163-179

Menjivar, J.A.; Rha, C.K.: Characterization of concentrated systems: constraint and compressibility effects. In: Rheology vol 2- Fluids. (Eds. Astarita, G.; Marrucci, G.; Nicolais, L.) Publd. Plenum Press, New York (1980) pp. 293-299

Meyle, J.; Gultig, K.; Brich, M.; Hammerle, H.; Nisch, W.: Contact guidance of fibroblasts on biomaterial surfaces. J. Mat. Sci: Mat. in Med. (1994) 5 463-466

Miekka, S.I.: Heat-induced fragmentation of human plasma fibronectin. Biochim et Biophys. Acta (1983) 748 374-380

Miller, W. M.; Peshwa, M. V.: Tissue engineering, bioartificial organs and cell therapies: I. Biotech. Bioeng. (1996) 50 347-348

Moncrieff, R.W. (Ed.) In: Man-made fibres. Publd. Heywood Books, London, (1969)

Moon, D.G.; Kaplan, J.E.: Fibronectin and blood coagulation. In: Fibronectin in Health and Disease. (Ed. Carsons, S.E.), CRC Prss, Inc, Florida, (1989) pp. 148-160

Mooney, D. J.; Organ, G.; Vacanti, J.P.; Langer, R.: Design and fabrication of biodegradable polymer devices to engineer tubular tissues. Cell Transplantation (1994) 3 (2) 203-210

Mooney, D. J.; Mazzoni, C.L.; Breuer, C.; McNamara, K.; Hern, D.; Vacanti, J.P.; Langer, R.: Stabilized polyglycolic acid fibre-based tubes for tissue engineering. Biomaterials (1996) 17 115-124

Morgenthaler, J.J.; Baillod, P.; Friedli, H.: Isolation of fibronectin under mild conditions. Vox. Sang. (1984) 47 41-46

Moria, A.; Zhang, Z.; Ruoslahti, E.: Superfibronectin is a functionally distinct form of fibronectin. Nature (1994) 367 193-196

Morrison, P.R.; Edsall, J.T.; Miller, S.G.: Preparation and properties of serum and plasma proteins. XVIII. The separation of purified fibrinogen from fraction I of human plasma. J. Am. Chem. Soc. (1948) 3103-3108

Mosesson, M. W.; Umfleet, R. A.: The cold-insoluble globulin of human plasma. J.B.C. (1970) 245 (21) 5728-5736

Mosesson, M.W.; Chen, A.B.; Huseby, R.M.: The cold-insoluble globulin of human plasma: studies of the essential structural features. Biochim. Biophys. Acta. (1975) 386 509-524

Mosesson, M.W.; Amrani, D.L.: The structure and biologic activities of plasma fibronectin. Blood (1980) 56 (2) 145-148

Mosher, D.F.: Cross-linking of cold-insoluble globulin by fibrin-stabilizing factor. J.B.C. (1975) 250 (16) 6614-6621

Mosher, D.F.: Physiology of fibronectin. Ann. Rev. Med. (1984) 35 561-575

Myers, R.; Wickerhauser, M.; Charamella, l.; Simon, L.; Nummy, W.; Brodniewicz-Proba, T.: Large-scale preparation of a highly purified solvent-detergent treated factor VIII concentrate. Vox. Sang. (1991) 60 141-147

Navsaria, H. A.; Myers, S. R.; Leigh, I. M.; McKay, I. A.: Culturing skin *in vitro* for wound therapy. TIBTECH (1995) 13 91-100

Nerem, R.M.: Tissue engineering in the USA. Med. & Biol. Eng. & Comput. (1992) 30 CE8-12

Nerem, R.M.; Sambanis, A.: Tissue Engineering: From biology to biological substitutes. Tissue Engineering. (1995) 1 (1) 3-13

Nieuwenhuizen, W.: Biochemistry and measurement of fibrinogen. Eur. Heart Journal (1995) 16 (Supplement A) 6-10

Oakley, C.; Brunette, D.M.: The sequence of alignment of microtubules, focal contacts and actin filaments in fibroblasts spreading on smooth and grooved titanium substrata. J.Cell. Sci (1993) 106 343-354

Osterlund, E.: The secondary structure of human plasma fibronectin; conformational changes induced by acidic pH and elevated temperatures; a circular dichroic study. Biochim. et Biophys. Acta (1988) 955, 330-336

Palecek, S.P.; Loftus, J.C.; Ginsberg, M.H.; Lauffenburger, D.A.; Horwitz, A.F.: Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. Nature (1997) 385 (6) 537-540

Parfentjev, I.A.; Johnson, M.L.; Cliffton, E.E.: The determination of plasma fibrinogen by turbidity with ammonium sulphate. Arch. Biochem. Biophys. (1953) 46 470-480

Pasqualini, R.; Bourdoulous, S.; Koivunen, E.; Woods, V.L.; Ruoslahti, E.: A polymeric form of fibronectin has antimetastatic effects against multiple tumor types. Nature Medicine (1996) 2 (11) 1197-1203

Perttila, J.; Salo, M.; Peltola, O.: Plasma fibronectin concentrations in blood products. Int. Care Med. (1990) 16 41-43

Peters, M.C.; Mooney, D.J.: Synthetic extracellular matrices for cell transplantation. Mat. Sci. Forum (1997) 250 43-52

Petersen, T.E.; Skorstengaard, K.; Vibe-Pedersen, K.: Primary structure of fibronectin: In Fibronectin (Ed. Mosher. D.F.) Publd. Academic Press Inc., London (1989) pp. 1-21

Petite, H., Frei, V., Huc, A., Herbage, D.: Use of diphenylphosphorylazide for cross-linking collagen based biomaterials. J. Biomed. Mater. Res. (1994) 28 159-165

Prajapati, R. T.; Al-Ani, S.; Smith, P.J.; Brown, R.A.: Fibronectin mats assessed in an *in vitro* model for keratinocyte grafting. Cellular Engineering (1996) 1 143-149

Procyk, R.; Adamson, L.; Block, M.; Blomback, B.: Factor XIII catalyzed formation of fibrinogen-fibronectin oligomers - a thiol enhanced process. Thrombosis Research (1985) 40 833-852

Prowse, C.: Kill and Cure. The hope and reality of virus inactivation. Vox. Sang. (1994) 67 (S3) 191-196

Raphael, M.; Rohani, S.; Sosulski, F.; Isoelectric precipitation of sunflower protein in a tubular precipitator. Canadian J. Chem. Eng. (1995) 73 470-483

Raphael, M.; Rohani, S.: Isoelectric precipitation of sunflower protein in an MSMPR precipitator modelling of PSD with aggregation. Chem. Eng. Sci. (1996) 51 4379-4384

Rault, I.; Frei, V.; Herbage, D.; Abdul-Malak, N.; Huc, A.: Evaluation of different chemical methods for cross-linking collagen gels, films and sponges. J. Mat. Sci - Mat. in Med. (1996) 7 215-221

Regnault, V.; Rivat, C.; Stoltz, J.F.: Affinity purification of human plasma fibronectin on immobilized gelatin. J. of Chromatography - Biomedical applications (1988) 432 93-102

Reilly, J.T.; McVerry, B.A.; Mackie, M.J.: Fibronectin in blood products - an in vitro and in vivo study. J.Clin Pathol. (1983) 36 1377-1381

Ross, M.; Reith, E. J.; Romrell, L.J.: The Integumentary system, In: Histology - a text and atlas (Ed. Kist, K.) Publd. Williams and Wilkins, Baltimore (1989) pp. 347-377

Ruoslahti, E.; Pierschbacher, M.D.: New perspectives in cell adhesion: RGD and integrins. Science (1987) 238 491-497

Rutter, G.H.: Requirements for safety and quality of intravenous immunoglobulin G preparations. J.Neurology, Neurosurgery and Psychiatry (1994) 57 S2-5

Saba, T.; Jaffe, E.: Plasma fibronectin, (opsonic glycoprotein): its synthesis by vascular endothelial cells and role in cardiopulmonary integrity after trauma related to reticulendothelial function. Am. J. Med. (1980) 68 577-594

Sabbagh W.: Development of an urothelial bio-artificial graft model. Msc thesis, University of London (1997)

Salonen, E.; Vartio, T.; Miggiano, V.; Stahli, C.; Tacacs, B.; Virgallita, G.; De Petro, G.; Barlatis, S.; Vaheri, A.: A rapid and highly sensitive solid-phase enzyme immunoassay specific for human fibronectin using a characterised monoclonal antibody. J. Immunological Methods (1984) 72 145-156

Sawada, K.; Moritaka, S.; Nakao, Y.; Yasumatsu, K.: Edible protein fibres. U.S. Patent 3806611 (1974)

Schwarzbauer, J.E.; Mulligan, R.C.; Hynes, R.O.: Efficient and stable expression of recombinant fibronectin polypeptides. Proc. Natl. Acad. Sci. USA (1987) 84 754-758

Scopes, R. K.(Ed.): Separation by precipitation. In: Protein purification - principles and practice. Publd. Springer-Verlag, New York, (1994) pp.71-101

Scott, D.L.; Bedford, P.A.; Walton, K. W.: The preparation of plasma fibronectin antigen and antiserum. J. Immunological Methods (1981) 43 29-33

Seachrist, L.: FDA receives praise, complaints over tissue regulation. Bioworld® Today 8(52) March 18 (1997)

Segeren, A.J.M.; Boskamp, J. V.: Protein fibers. U.S. Patent 4018903 (1977)

Senti, F. R.; Copley, M. J.; Nutting, G. C.: Fibrous from globular proteins. J. Phys. Chem. (1945) 49 192-211

Shapiro, L.; Cohen, S.: Novel alginate sponges for cell culture and transplantation. Biomaterials (1997) 18 583-590

Shih, Y.; Prausnitz, J.M.; Blanch, H.W.: Some characteristics of protein precipitation by salts. Biotech. Bioeng. (1992) 40 (10) 1155-1164

Sjoberg, B.; Eriksson, M.; Osterlund, E.; Pap, S.; Osterlund, K.: Solution structure of human plasma fibronectin as a function of NaCl concentration determined by small-angle X-ray scattering. Eur. Biophys. J. (1989) 17 5-11

Snyder, E.L.; Ferri, P.M.; Mosher, D.F.: Fibronectin in liquid and frozen stored blood components. Transfusion (1984) 24 53-56

Stathakis, N.E.; Mosesson, M.W.: Interactions among heparin, cold-insoluble globulin, and fibrinogen in formation of the heparin-precipitable fraction of plasma. J.Clin Invest. (1977) 60 855-865

Stathakis, N.E.; Mosesson, M.W.; Chen, A.B.; Galanakis, D.K.: Cryoprecipitation of fibrin-fibrinogen complexes induced by the cold-insoluble globulin of plasma. Blood (1978) 51 1211-1222

Steinbuch, M.: Protein Fractionation by ammonium sulphate, Rivanol® and Caprylic acid precipitaion. In: Methods of plasma protein fractionation. (Ed. Curling, J.M.). Publd. Academic Press, London, (1980) pp.57-74

Sternberg, M.M.; Kim, C.Y.: Process for preparing wet spun proteinaceous filaments. U.S. Patent 3956514 (1976)

Stol, M.; Adam, M.; Deyl, Z.: Biospecific affinity chromatography of plasma fibronectin on cross-linked gelatin microbeads. J. of Chromatography - Biomedical applications (1987) 419 308-314

Suzuki, S.; Matsuda, K.; Isshiki, N.; Tamada, Y.; Ikada, Y.: Experimental study of a newly developed bilayer artificial skin. Biomaterials (1990) 11 356-360

Swingler, G. R.; Lawrie, R. A.: Influence of coagulating bath composition on the texture of fibres spun from blood plasma. Meat Science (1977) 1 161-167

Szoka, P.: Body Shop of the Future: Biotech's tissue engineers. Bioventure view (1993) Aug/Sept

Tabor, E.; Buynak, E.; Smallwood, L.A.; Snoy, P.; Hilleman, M.; Gerety, R.J.: Inactivation of hepatitis B virus by three methods: treatment with pepsin, urea or formalin. J.Med.Virol (1983) 11 1-9

Taha, F. S.; Abbasy, M.; El-Nockrashy, A.S.; Shoeb, Z.E.: Countercurrent extraction - isoelectric precipitation of sunflower seed protein isolates. J.Sci. Food Agric. (1981) 32 166-174

Thomas, A.M.L.; Harding, K.G.; Moore, K.: The effect of alginate dressings on macrophage activation. Abstract - 7th Annual Meeting of European Tissue Repair Society, Koln, Germany, August 23-26 (1997)

Tombs, M.P.: Fluid aqueous protein compostions and food products prepared therefrom. U.S.Patent 3870801 (1975)

Tooney, N.M.; Mosesson, M.W.; Amrani, D.L.; Hainfeld, J.F.; Wall, J.S.: Solution and surface effects on plasma fibronectin structure. J. Cell. Biol. (1983) 97 1686-1692

Traill, D.: Vegetable proteins and synthetic fibres. Chemistry and Industry (1945) 58-63

Underwood, S.; Afoke, A. Brown, R.A.; MacLeod, A.J.; Dunnill, P.:The physical properties of a fibrillar fibronectin -fibrinogen material with potential use in tissue engineering. Bioprocess Eng. (1999) 20 239-248

Varani, J.; Inman, D.R.; Fligiel, S.E.G.; Hillegas, W.J.: Use of recombinant and synthetic peptides as attachment factors for cells on microcarriers. Cytotechnology (1993) 13 89-98

Virkar, P.D.; Hoare, M.; Chan, M.Y.Y.; Dunnill, P.: Kinetics of the acid precipitation of soya protein in a continuous-flow tubular reactor. Biotech. Bioeng. (1982) 24 871-887

Visser, J.; Oosthoek, R.H.J.; Groenweg, J.W.; Dijkstra, H.:Protein fibres. U.S. Patent 4118520 (1978)

Von Wazer, J.R.; Lyons, J.W.; Kim, K.Y.; Colwell, R.E. (Eds.) Viscosity and flow measurement - a laboratory handbook of rheology. Publd. Interscience Publishers, John Wiley and Sons, New York (1963) pp. 85-86

Vuento, M.; Vartio, T.; Saraste, M; von Bonsdorff, C.; Vaheri, A.: Spontaneous and polyamine induced formation of filamentous polymers from soluble fibronectin. Eur. J. Biochem. (1980) 105 33-42

Walczak, Z. K. (Ed.): Formation of synthetic fibres. Publd. Gordon and Breach Science Publishers Inc, London (1977)

West, S.M.; Guise, A.D.; Chaudhuri, J.B.: A comparison of the denaturants urea and guanidine hydrochloride on protein refolding. Trans. IChemE (1997) 75 Part C 50-56

Whitworth, I.H.; Brown, R.A.; Dore, C.; Green, C.J.; Terenghi, G.: Orientated mats of fibronectin as a conduit material for use in peripheral nerve repair. J.Hand Surg. (British and European Vol.) (1995a) 20B 4 429-436

Whitworth, I.H.; Terenghi, G.; Green, C.J.; Brown, R.A.; Stevens, E.; Tomlinson, D.R.: Targeted delivery of nerve growth factor via fibronectin conduits assists nerve regeneration in control and diabetic rats. Eur. J. of Neruoscience (1995b) 7 2220-2225

Whitworth, I.H.; Brown, R.A.; Dore, C.J.; Anand, P.; Green, C.J.; Terenghi, G.: Nerve growth factor enhances nerve regeneration through fibronectin grafts. . J.Hand Surg. (British and European Vol.) (1996) 21B 4 514-522

Wilkins, L. M.; Watson, S. R.; Prosky, S. J.; Meunier, S. F.; Parenteau, N. L.: Development of a bilayered living skin construct for clinical applications. Biotech. Bioeng. (1994) 43 747-756

Williams, E.C; Janmey, P.A.; Ferry, J.D.; Mosher, D.F.: Conformational states of fibronectin- effects of pH, ionic strength and collagen binding. J.B.C. (1982) 257 (24) 14973-14978

Wojciak, B.; Crossan, J.; Curtis, A. S. G.; Wilkinson, C.D.W.: Grooved substrata facilitate *in vitro* healing of completely divided flexor tendons. J. Mat. Sci: Mat in Med. (1995) 6 266-271

Wojciak-Stothard, B.; Curtis, A.S.G; Monaghan, W.; McGrath, M.; Sommer, I.; Wilkinson, C.D.W.: The role of the cytoskeleton in the reaction of fibroblasts in multiple grooved substrata. Cell Motility and Cytoskeleton (1995a) 31(2) 147-158

Wojciak-Stothard, B.; Madeja, Z.; Korohoda, W.; Curtis, A.; Wilkinson, C.: Activation of macrophage-like cells by multiple grooved substrata. Topographical control of cell behaviour. Cell Biol. Int Rep. (1995b) 19 485-490

Wojciak-Stothard, B.; Denyer, M.; Mishra, M.; Brown, R.A.: Adhesion, orientation and movement of cells cultured on ultrathin fibronectin fibers. In Vitro Cell. Dev. Biol. - Animal (1997) 33 110-117

Wood, E.J.; Raxworthy, M.J.: *In vitro* reconstruction of human skin. The Biochemist (1994) Feb/March 3-7

Yamada, K.M.: Fibronectin domains and receptors. In Fibronectin (Ed. Mosher, D.F.). Publd. Academic Press Inc., London (1989) pp. 47-121

Yamada, K. M.; Weston, J.A.: Isolation of a major cell surface glycoprotein from fibroblasts. Proc. Nat. Acad. Sci. USA (1974) 71 (9) 3492-3496

Yamada, K.M.; Akiyama, S.K.; Hasegawa, T.; Hasegawa, E.; Humphries, M.J.; Kennedy, D.W.; Nagata, K.; Urushihara, H.; Olden, K.; Chen, W.: Recent advances in research on fibronectin and other cell attachment proteins. J. Cellular Biochem. (1985) 28 79-97

Yang, J.; Lin, C.: Effects of various viscosity enhancers and pH on separating haem from porcine red blood cells. J. Sci. Food Agric. (1996) 70 364-368

Yannas, I.V. Tissue regeneration templates based on collagen-glycosaminoglycan copolymers. Adv. Polym. Sci. (1995) 122 219-244

Yannas, I.V.; Burke, J. F.: Design of an artificial skin. I. Basic design principles. J. Biomed. Mater. Res. (1980) 14 65-81

Yannas, I.V.; Burke, J.F.; Gordon, P.L.; Huang, G.C.; Rubenstein, R.H.: Design of an artificial skin. II. Control of chemical composition. J. Biomed. Mater. Res. (1980) 14 107-131

Yano, N.; Takahashi, H.; Hayasho, Y.: Process for producing fibers from natural protein of animal origin. U.S. Patent 3843803 (1974)

Yoshizato, K.; Yoshikawa, E.: Development of bilayered gelatin substrate for bioskin: a new structural framework of the skin composed of porous dermal matrix and thin basement membrane. Mat.Sci and Eng. (1994) C1 95-105

Young, R. H.; Lawrie, R. A.: Utilization of edible protein from meat industry by-products and waste. II The spinning of blood plasma proteins. J. Fd. Technol. (1974) 9 171-177

Young, R. H.; Lawrie, R.A.: Utilization of edible protein from meat industry by-products and waste. III. The isolation and spinning of protein from lung and stomach. J. Fd. Technol. (1975a) 10 453-464

Young, R. H.; Lawrie, R.A.: Utilization of edible protein from meat industry by-products and waste. IV. Studies on the physical and mechanical properties of spun fibrous products. J. Fd. Technol (1975b) 10 465-474

Ziabicki, A. (Ed.): Fundamentals of fibre formation. The science of fibre spinning and drawing. Publd. John Wiley and Sons, London (1976)