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LASER ACTIVATED TISSUE GLUES FOR USE IN LASER ASSISTED VASCULAR ANASTOMOSIS.

BY

DAVID JOHN MANDLEY BSc

A Doctoral thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of the Loughborough University of Technology

May 22, 1995

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Acknowledgements

I would like to thank the following people, without whom I should not have been able to complete this project:

- Professor Frank Wilkinson, for his supervision, guidance, patience and understanding.

- Mr G. Alban Davies F.R.C.S. of Tissuemed, who initiated the research and through the National Heart Fund and latterly Tissuemed, funded the project.

- Professor Dieter Oelkrug and his research students at the University of Tubingen for their help on the measurements of the optical properties of tissue.

- Dr. S.P. Allwork (Hon. Curator, British Heart Foundation Museum of Heart Disease), and Miokh Nohadani, who provided assistance in the histological preparation and interpretation of the laser anastomoses.

- Dr. David McGarvey, Dr. Andy Goodwin, Dr. Dave Worrall, and all currently in the Photochemistry Research Group at Loughborough University of Technology for their help and support.

- My parents, Arthur and Barbara Mandley, who have provided me with limitless moral and financial support during my studies.

- Mark and all the friends I’ve made during my time at Loughborough.

- And finally to Jane whose love, support and patience have been invaluable.
4.3.1.1 Argon ion laser. No bovine albumin or chromophore...... 125
4.3.1.2 Argon ion laser with bovine albumin. No chromophore... 126
4.3.1.3 Argon ion laser with bovine albumin and chromophore... 128
4.3.2 Variation in the composition of bovine albumin..................... 130
4.3.2.1 Argon ion laser with bovine albumin. No chromophore... 130
4.3.2.2 Argon ion laser with bovine albumin and chromophore... 131
4.3.3 Variation in the chromophore...................................................... 132
4.4 Discussion............................................................................................. 133

5 Bursting pressure study II. End-to-end anastomosis of porcine splenic arteries .............................. 139
5.1 Introduction .......................................................................................... 139
5.2 Materials and methods ........................................................................... 141
5.2.1 Bovine albumin ............................................................................ 141
5.2.2 Chromophore .............................................................................. 141
5.2.3 Measurement of bursting pressure ............................................... 141
5.3 Results .................................................................................................. 141
5.3.1 Argon ion laser with bovine albumin. No chromophore... 142
5.3.2 Argon ion laser with bovine albumin and Basic fuchsin .. 143
5.3.3 Argon ion laser with bovine albumin and Eosin Y .... 144
5.4 Discussion............................................................................................. 145
5.5 The in vitro reinforcement of end-to-end anastomosis using bovine serum albumin and bovine fibrin................................................................................. 148
5.5.1 Introduction ................................................................................ 148
5.5.2 Materials and methods ................................................................. 148
5.5.2.1 Bovine fibrin and albumin solutions ................................. 149
5.5.2.2 Chromophore .................................................................. 149
5.5.2.3 Measurement of bursting pressure ................................... 149
5.5.3 Results ........................................................................................ 149
5.5.3.1 Argon ion laser. No bovine albumin or chromophore... 149
5.5.3.2 Argon ion laser with bovine albumin and eosin Y............ 150
5.5.3.3 Argon ion laser with bovine albumin, bovine fibrin and eosin Y ........................................................ 153
5.5.4 Discussion................................................................................... 156

6 The photochemical fading of dyes- A possible end-point for laser assisted vascular anastomosis................................................................. 158
6.1 Introduction.......................................................................................... 158
6.1.1 Photochromic approach............................................................... 160
6.1.2 Thermochromics.......................................................................... 162
6.1.3 Photoreduction of dyes................................................................ 163
6.2 Experimental......................................................................................... 164
6.2.1 The photofading of Basic fuchsin. Quantum yield of fade determination ................................................................. 164
6.2.1.1 Results ............................................................................ 167
6.2.1.2 Discussion........................................................................ 169
Table of Tables

Physical changes taking place in tissue as a result of temperature........................................ 21

Important variables in the assessment of studies on laser assisted vascular anastomosis
.................................................................................................................. 27

A comparison of the best results for laser anastomosis of coronary arteries in vitro using Argon ion and Nd:YAG lasers ................................................................. 29

A comparison of the optimum results produced for arteriotomies repaired with Argon and Nd:YAG lasers, with and without chromophores .............................................. 35

The Kubelka-Munk absorption and scattering coefficients at the principle Argon ion and Helium Neon laser emission wavelengths ..................................................... 62

The results of investigations into the optical properties of human aorta performed by Keijzer ............................................................................................................. 74

Results of the optical properties of porcine aorta measured in this study, compared with the measurements of Yoon ................................................................................ 75

The decadic absorption coefficients of a series of chromophores at the two principle Argon laser emission wavelength ................................................................. 83

The mean temperature rises of a range of aqueous solutions of Basic and Acidic fuchsin irradiated at a power of 2 watts for durations of 30 and 40 seconds .......... 97

The similarities between temperature rises in matched solutions of Basic and Acidic fuchsin, irradiated at a power of 2 watts for 30 seconds ........................................ 98

The mean temperature rises observed in a range of highly absorbing solutions of chromophore irradiated at a power of 2 watts for 50 seconds .................................. 99

Mean temperature increases of highly absorbing chromophore solutions, irradiated at a power of 2 watts for 50 seconds ................................................................. 107

Quantum yield of fluorescence of aqueous solutions of chromophore obtained by ratio with standard compounds ................................................................. 110

Quantum yield of fluorescence of Acridine Orange, Eosin Y, Phloxin and Erythrosin obtained from the literature ................................................................. 118

Laser parameters employed during the fashioning of longitudinal arteriotomies..... 123
The burst pressures obtained for arteriotomies repaired by Argon laser at a range of laser powers .......................................................... 125

The burst pressures obtained for arteriotomies repaired by Argon laser with bovine albumin at a range of laser powers .......................................................... 126

The burst pressures obtained for arteriotomies repaired by Argon laser with bovine albumin and Basic fuchsin at a range of laser powers ............................................. 129

The burst pressures obtained for arteriotomies repaired by Argon laser studied as a function of bovine albumin concentration .................................................. 130

The burst pressures obtained for arteriotomies repaired by Argon laser with bovine albumin and Basic fuchsin, studied as a function of bovine albumin concentration .. 131

The burst pressures obtained for arteriotomies repaired by Argon laser with bovine albumin and four different chromophores. Eosin, Erythrosin, Basic fuchsin and Phloxin .................................................................................................................. 132

The burst pressure obtained for end-to-end anastomoses repaired by Argon laser with bovine albumin, studied as a function of laser power .............................................. 142

The burst pressures obtained for end-to-end anastomoses repaired by Argon laser with bovine albumin and Basic fuchsin, studied as a function of laser power ........ 143

The burst pressures obtained for end-to-end anastomoses repaired by Argon laser with bovine albumin and Eosin Y, studied as a function of laser power .................. 144

The burst pressures obtained for end-to-end anastomoses repaired by Argon laser, studied as a function of laser power .......................................................... 149

The burst pressures obtained for end-to-end anastomoses repaired by Argon laser with bovine albumin and Eosin Y .......................................................... 150

The burst pressures obtained for end-to-end anastomosis repaired by Argon laser with bovine albumin, bovine fibrin and Eosin Y ........................................................ 153

The quantum yield of fade of Basic fuchsin in human albumin, studied as a function of Ascorbic acid concentration .......................................................... 167

The quantum yield of fade of aqueous solutions of Eosin Y (6×10⁻⁵ mol/dm³), studied as a function of EDTA concentration .................................................. 173

The quantum yield of fade of aqueous solutions of Eosin Y (1.4×10⁻⁵ mol/dm³), studied as a function of EDTA concentration .................................................. 174
The quantum yield of fade of aqueous solutions of Eosin Y ($2.7 \times 10^{-5}$ mol/dm$^3$), studied as a function of EDTA concentration ........................................................ 174

The quantum yield of fade of aqueous solutions of Eosin Y ($3.5 \times 10^{-5}$ mol/dm$^3$), studied as a function of EDTA concentration ........................................................ 174

The quantum yield of fade of aqueous solutions of Eosin Y ($4.7 \times 10^{-5}$ mol/dm$^3$), studied as a function of EDTA concentration ........................................................ 175

The quantum yield of fade of aqueous solutions of Eosin Y ($5.9 \times 10^{-5}$ mol/dm$^3$), studied as a function of EDTA concentration ........................................................ 175

The quantum yield of fade of aqueous solutions of Eosin Y ($7.5 \times 10^{-5}$ mol/dm$^3$), studied as a function of EDTA concentration ........................................................ 175

The quantum yield of fade of aqueous solutions of Eosin Y ($9.8 \times 10^{-5}$ mol/dm$^3$), studied as a function of EDTA concentration ........................................................ 176

The burst pressures obtained for end-to-end anastomoses repaired by Argon laser with bovine albumin and Eosin Y, without reducing agent EDTA. Studied as a function of laser power ........................................................ 187

The burst pressures obtained for end-to-end anastomosis repaired by Argon laser with bovine albumin and Eosin Y, with reducing agent EDTA. Studied as a function of laser power ........................................................ 188
# Table of Figures

<table>
<thead>
<tr>
<th>Figure Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three and four level laser systems</td>
<td>12</td>
</tr>
<tr>
<td>Transitions involved in the carbon dioxide laser</td>
<td>15</td>
</tr>
<tr>
<td>Transitions involved in the argon ion laser</td>
<td>16</td>
</tr>
<tr>
<td>The interaction of light in a turbid media</td>
<td>17</td>
</tr>
<tr>
<td>Schematic diagram of a single photon timing apparatus</td>
<td>41</td>
</tr>
<tr>
<td>Schematic of a nanosecond flash photolysis apparatus</td>
<td>44</td>
</tr>
<tr>
<td>Diagram showing geometry of back-scattered light is measured as a function of</td>
<td>51</td>
</tr>
<tr>
<td>distance ( r ) from the axis of the incident beam</td>
<td></td>
</tr>
<tr>
<td>Schematic of the experimental arrangement used to measure the lateral light</td>
<td>56</td>
</tr>
<tr>
<td>diffusion</td>
<td></td>
</tr>
<tr>
<td>Schematic showing geometry used to measure the diffuse reflectance ( R_d )</td>
<td>57</td>
</tr>
<tr>
<td>Schematic showing geometry used to measure the diffuse transmission ( T_d )</td>
<td>58</td>
</tr>
<tr>
<td>The Kubelka-Munk absorption and scattering coefficients of porcine aorta, measured</td>
<td>61</td>
</tr>
<tr>
<td>as a function of wavelength</td>
<td></td>
</tr>
<tr>
<td>Normalised plot of the experimental radial intensity function ( 2\pi r J(r) )</td>
<td>63</td>
</tr>
<tr>
<td>for four different samples of porcine aorta</td>
<td></td>
</tr>
<tr>
<td>Normalised plot of the experimental radial intensity function ( 2\pi r J(r) )</td>
<td>64</td>
</tr>
<tr>
<td>for titanium dioxide, simulation and experimental</td>
<td></td>
</tr>
<tr>
<td>Normalised plot showing comparison between experimental radial intensity functions</td>
<td>66</td>
</tr>
<tr>
<td>( 2\pi r J(r) ), and a Monte Carlo simulated curve ( S=35 \text{cm}^{-1} ), ( A=1.1 \text{cm}^{-1} ), ( g=0.915 )</td>
<td></td>
</tr>
<tr>
<td>Normalised plot of Monte Carlo simulations obtained using identical optical</td>
<td>68</td>
</tr>
<tr>
<td>properties, ( S=40 \text{cm}^{-1} ), ( A=1.1 \text{cm}^{-1} ), ( g=0.915 ), but assuming</td>
<td></td>
</tr>
<tr>
<td>different boundary conditions, matched and mismatched</td>
<td></td>
</tr>
<tr>
<td>The absorption spectrum of oxyhaemoglobin</td>
<td>70</td>
</tr>
<tr>
<td>Normalised plot showing the difference between Monte Carlo simulations generated</td>
<td>76</td>
</tr>
<tr>
<td>by using the results obtained by a) reflectance and transmission and b) radial</td>
<td></td>
</tr>
<tr>
<td>reflectance measurements</td>
<td></td>
</tr>
</tbody>
</table>
Comparison of lateral light diffusion in porcine tissue and titanium dioxide.............. 79

Chemical structure and absorbance spectrum of Basic fuchsin. Concentration
3.5×10^-5 mol/dm3.................................................................84

Chemical structure and absorbance spectrum of Acidic fuchsin. Concentration
4.5×10^-5 mol/dm3.................................................................85

Chemical structure and absorbance spectrum of Acridine orange. Concentration
1.6×10^-5 mol/dm3.................................................................86

Chemical structure and absorbance spectrum of Carmine. Concentration
1.7×10^-5 mol/dm3.................................................................87

Chemical structure and absorbance spectrum of Crystal violet. Concentration
2.4×10^-5 mol/dm3.................................................................88

Chemical structure and absorbance spectrum of Eosin Y. Concentration
2.2×10^-5 mol/dm3.................................................................89

Chemical structure and absorbance spectrum of Erythrosin. Concentration
4.1×10^-5 mol/dm3.................................................................90

Chemical structure and absorbance spectrum of Neutral red. Concentration
7.8×10^-5 mol/dm3.................................................................91

Chemical structure and absorbance spectrum of Phloxin. Concentration
1.7×10^-5 mol/dm3.................................................................92

Chemical structure and absorbance spectrum of Ponceau. Concentration
4.7×10^-5 mol/dm3.................................................................93

Schematic showing the set-up used to measure temperatures generated as a result of
irradiating chromophores..........................................................95

The fading of Eosin Y as a result of irradiation, 2 watts, 50 seconds................. 100

The fading of Erythrosin as a result of irradiation, 2 watts, 50 seconds........... 101

The fading of Phloxin as a result of irradiation, 2 watts, 50 seconds.............. 102

The fading of Acridine orange as a result of irradiation, 2 watts, 50 seconds... 103

The temperature generating ability of Basic fuchsin as a function of dye
concentration........................................................................ 106
The fluorescence spectrum of Acridine orange and Rhodamine 590. (Inset shows wavelength dependency of fluorescence of Acridine orange) .................................. 111

The fluorescence spectrum of Eosin Y and Rhodamine 590. (Inset shows wavelength dependency of fluorescence of Eosin Y) ................................................................ 112

The fluorescence spectrum of Phloxin and Rhodamine 590. (Inset shows wavelength dependency of fluorescence of Phloxin) ................................................................. 113

The fluorescence spectrum of Erythrosin and Rhodamine 590. (Inset shows wavelength dependency of fluorescence of Erythrosin) .......................................... 114

The fluorescence spectrum of Acidic fuchsin and Rhodamine B. (Inset shows wavelength dependency of fluorescence of Acidic fuchsin) .................................... 115

The fluorescence spectrum of Basic fuchsin .................................................................. 116

Schematic showing the optical system coupling the argon and helium neon laser beams for effecting laser anastomosis .................................................................... 123

Schematic showing the experimental details for measuring the burst pressure of longitudinal arteriotomies repaired by laser ............................................................ 125

The absorption spectrum of bovine serum albumin ................................................ 128

Photograph of an artery repaired using an Eosin Y laser activated glue, using a power of 0.25 watts, evidence of thermal damage ............................................................ 147

Photograph of an artery repaired using an Eosin Y laser activated glue, using a power of 0.2 watts, no evidence of thermal damage ........................................................ 152

Photograph of an artery repaired using an Eosin Y/bovine fibrin laser activated glue, using a power of 0.25 watts, evidence of thermal damage ........................................ 154

Photograph of an artery repaired using an Eosin Y/bovine fibrin laser activated glue, using a power of 0.2 watts, no evidence of thermal damage .................................. 155

The photochemical ring closure of (E)-α-2,5-dimethyl-3-furyl-(ethylidene)isopropylidene) succinic anhydride ............................................................................. 161

Plot of quantum yield of fade of basic fuchsin ($4.8 \times 10^{-4}$ mol/dm$^2$) as a function of ascorbic acid concentration ............................................................................. 168

Plot of quantum yield of fade of Eosin Y as a function of dye and EDTA concentration ................................................................................... 177
Plot of the triplet state deactivation rate constant of Eosin Y (probed 570nm) as a function of dye concentration ................................................................. 180

Transient difference data traces for Eosin Y in aqueous solution for three different EDTA concentrations, probed 570nm ............................................................... 181

Fluorescence emission spectra of Eosin Y studied as a function of EDTA concentration ...................................................................................................................... 182

Photograph of an artery repaired using an Eosin Y laser activated glue, without EDTA, irradiated at a power of 0.3 watts. Showing evidence of thermal damage.. 190

Photograph of an artery repaired using an Eosin Y laser activated glue, without EDTA, irradiated at a power of 0.2 watts. No evidence of thermal damage .......... 191

Photograph of an artery repaired using an Eosin Y laser activated glue with EDTA, irradiated at a power of 0.3 watts. No evidence of thermal damage ............... 192

Photographs showing the difference in blood vessel appearance for arteries repaired using an Eosin Y laser activated glue at a laser power of 0.3 watts, with (right-hand), and without (left-hand) reducing agent EDTA ........................................ 193

A histological section through a laser anastomosis prepared using an Eosin Y laser activated adhesive, without EDTA, irradiated at a power of 0.25 watts. Elastic van Gieson stain x 40 ........................................................................................................ 198

A histological section through a laser anastomosis prepared using an Eosin Y laser activated adhesive, with EDTA, irradiated at a power of 0.2 watts. Haemotoxylin and Eosin stain x 40 ................................................................. 199

A histological section through a laser anastomosis prepared using an Eosin Y laser activated adhesive, with EDTA, irradiated at a power of 0.2 watts. Haemotoxylin and Eosin stain x 40 ................................................................. 200

A histological section through a laser anastomosis prepared using an Eosin Y laser activated adhesive, without EDTA, irradiated at a power of 0.2 watts. Elastic van Gieson stain x 40 ................................................................. 201

A histological section through a longitudinal arteriotomy repaired by laser, using a Basic fuchsin laser activated adhesive, without EDTA. Irradiated at a power of 0.2 watts. Haemotoxylin and Eosin stain x 40 ................................................................. 202

A histological section through a longitudinal arteriotomy repaired by laser, using a Basic fuchsin laser activated adhesive, without EDTA. Irradiated at a power of 0.2 watts. Haemotoxylin and Eosin stain x 16 ................................................................. 203
Abstract.

Laser assisted vascular anastomosis has been reported to have potential advantages over sutured anastomosis, such as reduced operating time, reduced foreign body reactions and lower potential for vessel constriction. The anastomosis of graft material during coronary bypass surgery is one possible use of laser anastomosis. Although laser anastomosis has achieved some success, a major limitation of the approach has been the lack of knowledge regarding the optimal laser exposure necessary to produce a consistent weld.

The first section of this thesis describes an investigation into the interaction of light within vascular tissue. Utilising the conventional Kubelka-Munk treatment of reflectance and transmission measurements, values of the absorption and scattering coefficients were obtained. Additionally, spatially resolved reflection measurements, (typically used for determining the optical properties of highly scattering homogenous samples such as silica and aluminium oxide), were performed on sections of porcine aorta to determine absorption, scattering and anisotropy coefficients. The suitability of a series of chromophores to enhance the absorption of laser radiation at the site of anastomosis have been assessed from the aspect of spectral profile, temperature generating ability, and determination of the fluorescence quantum yield.

The second section introduces the use of the Argon ion laser for tissue bonding. A range of in vitro experiments are detailed involving the repair of longitudinal arteriotomies and end-to-end anastomoses in porcine splenic arteries. These vessels have a similar wall thickness and diameter to human coronary arteries, furthermore, they have fewer side branches. An Argon laser was used with and without a range of chromophores and various protein additives. The use of a suitably absorbing chromophore and a reinforcing protein coagulant significantly increased the strength of blood vessel repair. Histological examination of the anastomotic sites, and the results of bursting pressure tests on end-to-end anastomoses are detailed.

The final section involved the development of chromophores whose absorption characteristics could be altered by irradiation. Steady state irradiation of various
compositions of dye/reducing agent has allowed determination of the quantum yields of photobleaching. Used in conjunction with a results of bursting pressure experiments, where the optimum irradiating conditions for successful bonding were determined, a system has been created whereby over-irradiation of blood vessels during anastomosis can be eradicated. The assessment of this system is presented. Histological examination and bursting pressure tests performed on end-to-end anastomoses of porcine splenic arteries are detailed. Nanosecond laser flash photolysis studies have been used to investigate the process of dye photobleaching. These studies have shown that the process of photofading is somewhat complicated, however, a mechanism involving the triplet excited state of the Eosin Y dimer has been proposed.
1. **INTRODUCTION.**

1.1 **Vascular anastomosis.**

Surgical anastomosis is a technique involving the closure of two tubular structures, such as blood vessels. An anatomical anastomosis leads to the fusion of two arteries, or an artery and a vein. The three commonly performed anastomoses are, end-to-end, end-to-side and side-to-side.

An anastomosis is the method employed in many areas of surgery, particularly vascular and cardiac, whereby biological or synthetic conduits are placed in such a way as to bypass the area of stenosis, narrowing or obstruction within the patient's own vessel \[^{1}\]. The two vessels most commonly employed as conduits are the saphenous vein and the internal mammary artery. The later is preferred as it demonstrates superior long term stability. End-to-side configurations are used in the majority of heart bypass operations.

Suturing anastomoses are commonly employed for vessel anastomoses. Such anastomoses are commonly fashioned by a continuous suture, sometimes performed with the aid of surgical magnifying loupes. The average operating time for sutured anastomosis is in the region of 10-15 minutes.

Needle trauma and the resulting foreign body reactions with suturing materials are associated with conventional sutured anastomosis \[^{2}\], this potentially leads to a loss of patency of the repaired vessels. The quality of artery grafting in coronary bypass surgery depends on a number of factors. Primarily the apposition of the two vessel edges prior to suturing. The onset of stenosis at the site of anastomosis within the first two months following surgery can frequently be attributed to manual inexactitude, particularly when speed of the operation is important. The time taken to perform vascular anastomoses varies depending upon the site, diameter of the vessels being joined, the surgical technique and the individual surgeons ability. Anastomotic time may be significant particularly in cardiac surgery where multiple anastomoses may be performed. Quicker and accurate anastomoses may reduce the time of patient is on the heart lung machine, with the associated morbidity and mortality. It is
intended to develop the technique of laser assisted glue anastomosis to enhance surgical results.

In an attempt to overcome the problems associated with suturing anastomoses and to reduce operating times, a number of alternative techniques have been suggested. One such procedure is laser vascular anastomosis. As a natural progression from laser assisted vascular anastomosis, (whereby vessels are primarily glued by thermal coagulation of their walls), this thesis describes the use of a laser activated glue as an adhesive to perform anastomoses. Laser assisted vascular anastomosis has met with difficulties associated with reduction in bursting pressures, aneurysm formation and anastomotic haemorrhage, as the body’s host cells repair the thermal damage, thus weakening the strength of the anastomosis over a period of time.

This section outlines the anatomy of blood vessels, considers the problems encountered with conventional sutured anastomosis, and discusses the use of alternative anastomotic techniques.

1.1.1 The circulatory system.

Upon leaving the heart blood enters the vascular system, which consists of numerous blood vessels. These vessels transport blood to all parts of the body, allowing exchange of nutrients, metabolic end products, hormones and other substances with the blood and the interstitial fluid. The blood then returns to the heart. Large blood vessels called arteries carry blood away from the heart. The large arteries then divide into smaller arteries, and then into smaller arteries and finally into capillaries. Capillaries merge into small vessels called venules, which lead into larger vessels known as veins. Larger veins transport blood back to the heart.

1.1.2 General structure of blood vessel walls

There are functional differences between blood vessels and their walls vary in thickness. The variation is due to the presence or absence of one or more of three layers of tissue which have differing thicknesses, for example smooth muscle and elastin.
The tunica intima.
The tunica intima is the innermost layer and comprises of a layer of polygonal endothelial cells, elongated in the direction of the blood flow. This endothelium lies on a layer of connective tissue consisting of a basal lamina consisting of type IV collagen microfibrils. Beyond the basal lamina is a subendothelial layer consisting of networks of collagenous microfibrils (type IV) and fibrils (type I, III), connective tissue cells, elastic fibres and smooth muscle cells embedded in amorphous ground substances.

Tunica media.
The middle layer, the tunica media is generally quite thick and is composed of varying quantities of connective tissue; collagenous microfibrils (type IV), fibrils (type I, II and V) and reticular fibres (type III), and elastic fibres. Generally the tunica media consists of these elements arranged in a tight spiral, and they appear circular. Typically, there is a layer separating the tunica intima and the tunica media. This is known as the internal elastic lamina and consists of a thin layer of elastic fibres. Additionally, between the border of the tunica media and the outermost layer of the blood vessel wall, there is a distinct layer of elastic fibres, the external elastic lamina. Generally it is the structure of the tunica media that determines the physical properties of the vessel wall.

Tunica adventitia (cor externa).
The outermost layer consists of a relatively thin layer of connective tissue comprising of orientated collagen (type I) and elastic tissue. They run parallel to the long axis of the vessel, and this arrangement enhances the mechanical strength of the blood vessel.

1.1.3 Structure of arteries.
The general structure of arteries is dependant on the function of the particular artery.
Elastic arteries.
Large arteries such as the aorta, its major branches and the pulmonary track are examples of elastic arteries. The walls of such vessels consist of the three definite layers described previously. The vessel wall is thin relative to the size of the lumen, when compared to muscular arteries. The subendothelial layer within the tunica intima contains elastic fibres and a high proportion of elastin is present in the media. This gives the artery a yellow appearance. The tunica media of large arteries is very thick and consists of elastic tissue arranged as concentric sheets connected to each other by fibrils. Distributed between the elastic fibres are collagen and smooth muscle cells.

Muscular arteries.
Typically muscular arteries are medium sized arteries. These arteries are characterised by a tunica media containing a considerably higher proportion of smooth muscle cells to elastic fibres. The thickness of the wall in proportion to the vessel diameter is the greatest of the blood vessels.

Veins.
Veins receive blood from the venules. They possess the same three coats as arteries. However, the middle layer, the tunica media is thinner and has considerably fewer muscle fibres. A prominent feature of veins is that the tunica externa forms the greatest part of the wall, and is several times thicker than the media. However veins have no internal, or external elastic lamina. Veins have a larger calibre lumen and thinner walls than the comparative arteries. The distinguishing feature of veins when compared to arteries, is that some veins contain valves that only allow blood to flow towards the heart. These valves are as a result of folds of the tunica intima. As a consequence to the pull of gravity, blood within veins flows backwards away from the heart. The flaps of the valves fill with blood and prevent retrograde blood flow.
1.1.4. **Collagen**

Collagen is a rigid and inextensible protein and is the major constituent of tendons and other connective tissue. Collagen constitutes 30% of the dry weight of the body. There are a number of different types of collagen, with different chemical compositions. The polypeptide chains of collagen have a complex helical structure. The collagen helix is unique, it is a left handed helix. Analysis of collagen amino acid sequences shows them to be characterised by a repetitive tripeptide sequence of Gly-X-Pro or Gly-X-Hydroxyproline, where Gly is glycine, Pro is proline and X is any amino acid. Collagen is 35% glycine, 11% alanine and 21% proline and hydroxyproline. The collagen molecule is known as the tropocollagen molecule. It is composed of three left handed polypeptide helices coiled around each other to form a right handed supercoil. The polypeptide make-up determines the type of collagen produced. Collagen I, the most common type of collagen is composed of two kinds of polypeptide helices, $\alpha_1$ and $\alpha_2$. These combine in a 2:1 ratio respectively to form a type I triple helix. Because each of the chains of tropocollagen is a helix with three residues per turn, every third amino acid residue of a given strand makes unusually close contact with the other two strands. The close contacts that occur along the central axis of the triple helix leave room only for the hydrogen atom side chain of glycine, any bulkier side chain would push the three strands of tropocollagen apart.

An increase in temperature alters the triple helical structure of collagen resulting in a random coil structure. This process is known as denaturation. It is a familiar process that occurs when an egg is cooked. Egg white, containing soluble protein egg albumin coagulates to a white solid on heating. The egg white will not redissolve on cooling to give a clear solution of protein. Consequently, heating of egg albumin changes its structure irreversibly. This effect occurs with most globular proteins, and it is independent of molecular structure, size or biological function. The temperature at which the transition occurs in collagen has been reported as 68°C [3] and 65°C [4]. Even allowing for minor variations, these temperatures are in a similar region recorded for the heat denaturation of arteries and veins, the process required during laser vascular anastomosis. (75°C and 70°C [5] respectively). In addition to heat,
collagen can be denatured by changes in pH and by certain organic solvents such as alcohol and acetone, and even solutions such as urea.

1.1.5. Elastin.
Tissue within the body such as blood vessels and the skin must be elastic. The flexibility of these structures is provided by a specialised protein known as elastin. Elastin has a molecular weight of between 64000 and 66000. In comparison to collagen, where rigid polypeptide chains are evident, elastin has an irregular, random coil conformation. Like collagen, elastin is rich in glycine and proline, but contains no hydroxylysine and hydroxyproline. Elastin fibres are formed when the side chains of adjacent polypeptides become covalently cross linked. Crosslinking between elastin polypeptides can occur in two ways:

1. Oxidation of the side chains of a lysine residue, followed by condensation of the aldehyde group with the ε-amino group of another lysine residue. This reaction is also observed in collagen crosslinks.

2. Condensation of three allysine residues with the ε-amino group of a lysine residue to give a product known as desmosine. This is simply a molecular knot that can tie together four chains of elastin.

The elasticity of the large elastic arteries stores energy within the vessel wall during contraction. Release of this energy during expansion contributes to the maintenance of diastolic blood pressure.

1.2. Alternative anastomotic techniques.

While the occurrence of vessel trauma is a cause for concern, conventional sutured anastomosis is time consuming, and requires the utmost precision by the operator. To overcome the problems associated with suturing a number of different techniques have been investigated. The use of mechanical devices to replace the use of sutures for vascular surgery have been reported as long ago as the late 19th century. Such devices are categorised depending upon the type of joint they generate, butt, flanged or lapped. Flanged joints are the types of joints produced when vascular anastomosis
are prepared by evertting sutures. These early devices proved no more successful than existing techniques. Devices to generate lapped joints have been described by a number of different investigators [7,8,9]. Payer [9] used a absorbable Magnesium ring and the opposed vessel ends were supported using a silk ligature. Similarly hooks and pins have been used to support vessels [7].

Instruments to prepare flanged joints have been developed [10,11]. Androsov [10] used a stapling device for the anastomosis of bloods vessels with diameters ranging from 1.3 to 15mm. Patent anastomoses were reported in 66 patents, however, the long term quality of the anastomoses were not studied. An alternative technique has involved the use of soluble intravascular stents placed inside the vessels prior to anastomosis. Tibbs [12] used a diamond shaped ice cube and Ballinger [13] a solidified dextrose rod. More recently, studies have involved the use of intraluminal stents with tissue sealants [14,15]. Yamagata [14] used a polyvinyl alcohol (PVA) stent (to support the anastomoses and prevent the vessel walls from collapsing) with a cyanoacrylate based cement for the in vivo end-to-end anastomosis of rat carotid arteries. Similarly a polyethylene glycol stent and bovine fibrinogen based glue [15] were used for the microvascular end-to-end anastomosis of rat femoral arteries and veins. A patency rate of 100% was reported, with all anastomoses able to tolerate an intra vascular pressure of 250cm H₂O.

Later studies have concentrated on the use of various types of tissue adhesives. Conflicting results have been reported [16,17,18]. Silverstein [16] used a fibrin glue for the vasovasostomy in rats. Fibrin glue repairs were compared with conventional sutured vasovasostomy. The use of fibrin glue allowed the preparation of a sperm tight anastomosis, that had the advantage of reducing the operating time and required less surgical skill. Seguin [17] performed 42 Type A aortic dissections. Three different groups were assessed, in group one the ascending aorta was replaced with an intraluminal graft. Group two consisted of a Dacron prosthesis sutured to the aorta, and in group three the proximal and distal aortic stump were glued together and reinforced at the sites with a fibrin based adhesive before implantation of the Dacron
prosthesis. The comparative study revealed that the intraluminal grafts for replacing the ascending aorta in cases of aortic dissections demanded a high degree of surgical skill. However the use of a fibrin sealant reduced mortality, post operative bleeding and improved results of operation for acute aorta dissections. However, Van der Ham[118] expressed doubts over this technique, suggesting that fibrin sealants have detrimental effects on the quality of anastomosis. Colonic anastomoses of rats were studied using three different experimental techniques. Group one consisted of animals that underwent sutured colonic anastomoses, group two used sutured anastomoses with human derived tissue, and group three was used as a control group, taking into account possible reactions with foreign protein. Colonic anastomoses were repaired using sutures and a specially prepared rat fibrin adhesive. All animals were sacrificed within the first seven days following surgery. Results of bursting pressure and hydroxyproline concentration (to determine collagen concentration and degradation) were studied, it was concluded that fibrin sealing of the colonic anastomoses in the rat does not improve healing, and appeared to have a negative influence.

Attempts to reduce the extent of vessel trauma and minimise operative time by the methods previously described have achieved differing rates of success. The use of lasers to fashion vascular anastomosis will be assessed under the same criteria.

1.3. **LASERS.**

The term laser is an acronym for Light Amplification by Stimulated Emission of Radiation. Laser action relies on the principle of basic quantum mechanics applied to the absorption and emission of light. Before detailing the mechanism of laser action, it is important to describe some of the properties of lasers that enable their use for a variety of applications, including medical devices, and as excitation sources in photochemical studies.

**Directionality**- Laser sources are highly directional sources with a minimal divergence. The directionality of laser light is due to the fact that within the laser
resonant cavity, only one electromagnetic wave propagating along the cavity direction (or in a direction very near to it) can be sustained in the cavity.

**Coherence**- Lasers are coherent light sources. Coherence is a property resulting from the nature of the stimulated emission process. Photons generated by a laser have the same frequency and are emitted in phase with one another, whereas light produced by conventional light sources, by spontaneous emission is chaotic, since there is no correlation between the phases of different photons.

**Monochromaticity**- Monochromaticity is a feature of laser light that is most relevant for its use in chemistry, it is caused by the fact that all the photons are emitted as a result of a transition between the same two energy levels and therefore possess identical frequencies.

Laser action depends upon two features: light amplification by stimulated emission, and optical oscillation in a resonant cavity.

1.3.1. **Light amplification by stimulated emission.**

Consider an atomic system comprising of two energy levels, n, the upper level, and m the lower level. The energies and populations of these energy levels are $E_n$, $E_m$ and $N_n$, $N_m$ respectively. One process that can take place in this case is the absorption of a photon of light, possessing energy $E_{nm}=E_n-E_m$. This results in the promotion of an atom from the lower level to the upper level. As a result of absorption of radiation, there are two possible radiative processes that can take place, spontaneous emission and stimulated emission. Photons generated by spontaneous emission have random phase and the light produced is known as incoherent light. If a system contains a number of molecules in an excited state, and a beam of light is directed into the system with a frequency that the photons energy matches the gap between the excited and lower energy state, a molecule can be stimulated to emit a photon with the same frequency as the stimulating radiation. The process is referred to as stimulated emission.
If the beam of emitted photons becomes more intense, or is lost, is dependant on which of photon absorption or stimulated emission dominates the system. We require a greater number of atoms in the upper state than in the lower state. This is known as a population inversion. A population inversion can be achieved by two techniques;

- Optical pumping, where the lasing medium is surrounded by a flash tube that emits light with a frequency required to excite the lasing material.

- An electric discharge, resulting in ionisation and acceleration of charged particles in an electric field. Accelerated particles collide with particles of the laser medium and result in excitation.

Optical pumping is used in solid state lasers such as the Nd:YAG laser. Alternatively, an electrical discharge is used in gas lasers such as the Argon ion laser.

![Diagram of Ruby laser and Nd:YAG laser level structures](image)

Figure 1.1: Three- and Four-level laser systems

Pumping is not the only requirement for laser action. The disadvantage of using a two level system, described previously, is that absorption and stimulated emission may
be triggered by the same photon. Ultimately the population of the upper and lower energy levels are equal, and population inversion is not achieved. Consequently 3 or 4 level systems are used. The ruby and Nd:YAG lasers are examples of 3 and 4 level lasers, the transitions involved in the laser action are shown in figure 1.1. For the ruby laser the population inversion results from pumping a majority of Cr$^{3+}$ ions into an excited state, followed by a radiationless transition to another excited state. The transition from this level to the ground state is the laser transition, and results in red 694nm radiation. The Nd:YAG laser is a 4 level system. The laser transition is between two excited states. The advantage of such a system is that as the population inversion is between two excited states, it is much easier to achieve than between an excited state and an intensely populated ground state.

**Optical oscillation in a resonant cavity.**

The optical cavity is necessary to produce a directional beam to give the beam coherence and to maintain laser action. The use of a fixed cavity length means that only light waves that have nodes at the mirror surfaces will be reflected back along the original light path with minimal losses. Maximum amplification can only be achieved for the light waves that satisfy this ‘standing wave’ condition. These light waves are characterised since they have wavelengths that satisfy the condition,

$$\lambda = \frac{2d}{n}$$  \hspace{1cm} (1.1)

where $d$ is the cavity length and $n$ is the number of half wavelengths contained within the cavity. These light waves are called the axial or longitudinal cavity modes.

**1.3.2. Lasers used for vascular anastomosis.**

Since the introduction of laser assisted vascular anastomosis by Jain and Gorisch$^{119}$, a number of lasers have been used. The three most widely used lasers are described in more detail in the following sections.
1.3.2.1. Neodymium-Yttrium Aluminium Garnet laser (Nd:YAG).

The Nd:YAG laser is composed of an active medium of yttrium aluminium garnet doped with neodymium. The transitions in the neodymium ions account for the laser action (see figure 1.1), however, the wavelength of emission is affected by the presence of different host lattices. Pumping of neodymium lasers is achieved by a flashlamp, however, more recently, Nd:YAG lasers utilising a diode laser as the pumping system have been developed. The typical output power of a continuous wave Nd:YAG laser lies in the region of 100 watts. However as the efficiency of such lasers is only 1%, large amounts of electrical energy are required.

1.3.2.2. Carbon dioxide laser.

The CO₂ laser has one of the highest working efficiencies of all common lasers. The laser medium contains a mixture of three gases, carbon dioxide, helium and nitrogen. Helium is used to improve the laser efficiency and nitrogen enables energy transfer to carbon dioxide (see figure 1.2). Pumping of the carbon dioxide laser is provided by a high voltage electric current. Powers used for surgical applications of carbon dioxide lasers range from 20-40 watts.
Figure 1.2: The transitions involved in the carbon dioxide laser.

1.3.2.3. Argon laser.

Argon lasers consist of a plasma tube containing Argon gas at a pressure of approximately 0.5mbar. Excitation is provided by an electrical discharge. Powers of between 10-20 watts are readily obtainable from commercial devices, however because the efficiency of such lasers is only 0.1%, large amounts of electrical energy are required to produce output powers within this range. As a result of the poor energy conversion of inert gas lasers, vast amounts of thermal energy are generated, therefore the cooling capacity is an important criteria in the design of argon lasers. An energy level diagram showing the important transitions of the argon laser are shown in figure 1.3.
Figure 1.3: The interactions involved in the Argon ion laser.

1.4. Laser tissue interactions.

The possible interactions of light and tissue are shown in figure 1.4. A small percentage, typically 4% of the incident laser beam is reflected from the surface as a result of a mismatch in the refractive index. Such a reflection is known as Fresnal reflection. The remaining radiation is transmitted into the tissue. Some of this light may be scattered. Fractions of the collimated and scattered light within the tissue are absorbed. The extent to which these processes occur is affected by the optical properties of the irradiated tissue, and the wavelength of laser radiation.
1.4.1. Absorption.

When a photon of light is absorbed by a molecule, the energy is transferred from the photon to the molecule, resulting in annihilation of the photon. Upon absorption of the photon, excitation of an electron in the molecule ensues. When the electron undergoes the transitions back to its lowest energy levels, the excited energy decays non-radiatively to produce thermal energy. Certain materials possess specific absorption characteristics, whereby particular wavelengths of light are absorbed to a greater degree. This phenomenon leads to the characteristic absorption spectrum.

When scattering of light does not occur, the linear absorption of electromagnetic radiation is governed by the Beer Lambert law, which relates the transmitted monochromatic intensity $I$ through a sample of thickness $l$, to the incident intensity $I_0$, according to, $I = I_0 e^{-\alpha l}$. It can be seen that the intensity decreases exponentially with the sample thickness and concentration. The Beer Lambert law is often written as,
I = I₀ 10⁻εc₁ or \( \log I/I₀ = -\varepsilon c₁ \)  \hspace{1cm} (1.2)

\( \varepsilon \) is known as the molar absorption coefficient of the species at the stated wavelength, and is related to \( \alpha \) by \( \varepsilon = \alpha \ln 10 \). The value of the molar absorption coefficient depends upon the molecule and the frequency of light.

1.4.2. Reflection.

Reflection occurs when light passes through interfaces which separate media of two different refractive indices. There are two different types of reflection, diffuse or specular. If the surface of a material is rough to such an extent that the roughness is a similar order of magnitude or greater than the wavelength of the incident light, diffuse reflection occurs. Specular reflection occurs when light is reflected back at an angle identical to that of the incident beam.

1.4.3. Scattering.

Biological tissue, particularly non homogeneous tissue usually involve a certain degree of light scattering. Consequently the internal photon flux gradient is not only affected by absorption. At laser wavelengths where scattering is significant (the visible and near infra red wavelengths), the Beer Lambert law is not applicable and the Kubelka-Munk theory should be used \(^{201}\). In such instances the equation describing the laser intensity \( I(z) \) into a tissue sample is,

\[
I(z) = I₀ \exp[\varepsilon (A+S)z] \hspace{1cm} (1.3)
\]

where \( I(z) \) is the laser intensity at position \( z \) (mm) below the tissue surface (watts/cm²), \( I₀ \) is the incident laser intensity (watts/cm²), \( \varepsilon \) is the absorption coefficient (cm⁻¹), \( S \) is the scattering coefficient (cm⁻¹) and \( z \) is the penetration depth (mm).

If light is scattered by particles smaller than the wavelength of the incident light, the beam is dispersed away from the path of the light beam. This type of scattering is known as Rayleigh and is commonly seen at shorter wavelengths, particularly in the
ultraviolet region. Light scattered as a result of Rayleigh scattering does not change wavelength, compared with Raman scattering. If the scattering particles are larger in size than the wavelength of light, for example, green light, 0.4\(\mu\)m in diameter, the scattering is called Mie scattering. In comparison to Rayleigh scattering, Mie scattering is predominantly forward directed. The extent to which scatter occurs is dependent on the density of the inhomogeneities, their size and shape, as well as the refractive index of the tissue and the wavelength of the radiating source. If the penetration depth of the laser is sufficient, scattering produces the following effects.

* As a result of reabsorption of scattered laser light, volumetric heating of the tissue ensues.

* Misleading estimates of the absorption coefficient and the possible heating effects of the laser, due to the backward and forward scattering of the incident light

* A phenomenon known as the 'popcorn effect'\(^{[21]}\).

Kroy\(^{[22]}\) suggested that as a result of reabsorption of scattered light, higher temperatures occur inside the tissue rather than at the surface. The difference caused by scattering can be dramatically demonstrated by the Nd:YAG irradiation of egg white\(^{[23]}\). Coagulation occurs at the surface because no scattering is present in the transparent phase. However, as a result of continued irradiation it is commonplace for the coagulated bud to explode, indicating that the boiling temperature is first attained at a depth below the surface. This feature is explained in the paper of Keijzer\(^{[24]}\) who argues that the maximum radiation intensity in scattering media is not at the irradiated surface but at an interior point of the medium. Typically for Nd:YAG radiation, the depth of the maximum can be up to half a millimetre, while for the CO\(_2\) laser which is more strongly absorbed by tissue, the maximum intensity is much nearer the surface.
1.4.4. Transmittance.

The proportion of incident light passing through a material for a particular distance is known as the Transmittance. It can be defined by the Beer-Lambert law as,

\[ \frac{I}{I_0} = 10^{-\alpha d} \]  

(1.4)

1.4.5. The wavelength dependency of absorption and scattering processes.

The ultra violet region of the absorption spectrum shows high absorbance, due principally to the occurrence of biomolecules such as proteins and nucleic acids. Nucleic acids, which constitute 10-15% of a cell's dry weight are the most widespread absorbers in the 190-300nm wavelength region. As the wavelength increases, the absorption decreases and the absorption as a result of particular chromophores, such as bilirubin and haemoglobin dominates. Haemoglobin, contained within red blood cells is the most essential element in the transportation of oxygen around the body. Haemoglobin exhibits a strong absorption band at 420nm. The oxygenated form of haemoglobin possesses two absorption bands at 540 and 580nm. As a result of selective absorbances by specific molecules, it would be expected to see different absorbances among different tissue types. As the wavelength increases into the infra red region, the absorption by biological materials is dominated by water.

Generally, the scattering coefficient increases with a decrease in particle size. There is little structure associated with the scattering spectrum of tissue given the range of potential scatterer sizes. However, it is generally accepted that the extent of scatter decreases with increasing wavelength.

1.4.6. The photothermal effects of laser radiation.

On absorption of laser energy, a high proportion is rapidly converted to heat by a non-radiative process. Since laser light is characterised by its minimal beam divergence a small spot can be employed, resulting in confined heating. If water is the absorbing chromophore the tissue undergoes rapid dehydration as the water is converted to
steam. The first mechanism by which tissue is thermally affected is molecular denaturation, of, for example proteins, collagen, lipids, haemoglobin. Table 1.1 summarises the temperatures at which particular transformations occur.\cite{25}

Conversion of electromagnetic radiation into heat

\[ \downarrow \]

Elevation of tissue temperature.

<table>
<thead>
<tr>
<th>Tissue temperature/°C</th>
<th>Change occurring to the blood vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td>43-50</td>
<td>Uncoiling of collagen helices</td>
</tr>
<tr>
<td></td>
<td>Reversible tissue denaturation</td>
</tr>
<tr>
<td></td>
<td>Reduction of enzyme activity</td>
</tr>
<tr>
<td>60</td>
<td>Irreversible protein denaturation</td>
</tr>
<tr>
<td></td>
<td>(continued irradiation results in cell damage)</td>
</tr>
<tr>
<td>80</td>
<td>Collagen, elastin, proteoglycan degradation</td>
</tr>
<tr>
<td>100</td>
<td>Boiling of tissue cell water</td>
</tr>
<tr>
<td>150</td>
<td>Pyrolysis</td>
</tr>
<tr>
<td>200</td>
<td>Carbonisation</td>
</tr>
<tr>
<td>500</td>
<td>Ablation of calcified tissue</td>
</tr>
</tbody>
</table>

Table 1.1. Physical changes taking place as a result of temperature rises.\cite{25}

As biological tissue is heated from 43-50°C, the collagen helices uncoil, resulting in reversible tissue denaturation. If the heating is not prolonged, cooling of the tissue results, and the coils may fuse together. This process is the principle involved in laser assisted vascular anastomosis. However, controlling blood vessel temperatures in the region of 50°C is extremely difficult, considering the number of variables involved in laser welding. As the temperature of the tissue increases beyond 60°C, irreversible
protein denaturation occurs. If the tissue is exposed to these temperatures for a short time, the cells are injured. Prolonged exposure results in cell death. As the temperature of tissue increases beyond 80°C, collagen, elastin and proteoglycans, the principle structural elements of tissue begin to degrade. At 100°C, water in tissue cells boils, this results in a potentially explosive release of steam. When tissue temperatures increase beyond 150°C, pyrolysis of the tissue is observed. At temperatures above 200°C, the tissue begins to carbonise. If required continuous wave lasers can be used to ablate calcified tissue, such a feature is used in laser angioplasty. Temperatures in excess of 500°C are required for such applications. Laser ablation serves as the basis of all photosurgical or photocoagulative applications.

The major problem with the use of lasers for surgical procedures is to adjust the duration of laser exposure so to reduce the incidence of tissue damage to surrounding areas and to obtain limited necrosis. The determining factor for this problem is known as the thermal relaxation time, $\tau$. This value indicates the maximum time for which heat can be delivered whilst still maintaining an effect localised to the irradiated zone.

Ultraviolet laser energy can be used for typical surgical procedures, particularly for use in tissue ablation. Ultraviolet photons contain sufficient energy to break molecular bonds. This process is more efficient than the thermal effects of laser radiation because the laser energy is employed directly in bond breaking. The principle ultraviolet absorbing chromophores within tissue are proteins or lipids. Pulsed ultraviolet radiation leads to the breaking of bonds in these large molecules, which are converted to smaller fragments and are ejected from the surface of the material. The ejection of these smaller molecules removes much of the energy used to break the chemical bonds. Because the pulse duration is less than 500 nanoseconds, (which is below the 'thermal relaxation time' for typical tissue), there is little time for radiative or conductive heat transfer to take place. Consequently, there is no damage to the surrounding tissue. For this process to be efficient, the tissue must be a
reasonable absorber. Vascular tissue consists mainly of water, organic compounds and a few inorganic salts. The proteins and lipids within blood vessels strongly absorb ultraviolet light and are ideal chromophores.

1.4.7. Non-thermal effects of laser radiation.

1.4.7.1 Photochemical processes.

A number of different photochemical reactions have been used with medical applications. One beneficial effect of ultra violet radiation has been the production of vitamin D$_3$ following absorption of light by human skin. The conversion of 7-dehydrocholesterol into vitamin D$_3$ is a two step process. First, 7-dehydrocholesterol absorbs radiation at wavelengths shorter than 320nm and changes into previtamin D$_3$. This reaction occurs throughout the epidermis. Second, previtamin D$_3$ thermally isomerises to form vitamin D$_3$ which binds preferentially to the vitamin D binding protein in capillaries. If there is excessive cell injury to the skin as a result of the absorption of ultra violet light, the whole organ reacts. After a period of several hours, redness occurs and is followed by tanning and thickening. ‘Sunburn’ is an example of a generalised, primitive, protective, pathophysiologic response designed to remove injurious agents, and many of its features are similar to those of reactions caused by other irritating or toxic agents. An example of an in vivo photochemical reaction is the blue light phototherapy of hyperbilirubinemia [26]. Children born prematurely may have inadequate enzyme activity in order to convert bilirubin from a toxic lipophilic metabolite to a water soluble form which can be excreted into the intestine. The consequences of high bilirubin levels in blood and tissue include irreversible damage to the central nervous system. Exposure of children to banks of fluorescent visible lamps results in a reduction of bilirubin levels. Bilirubin absorbs blue light, and is photodegraded to smaller units. The most likely mechanism for explaining phototherapy of hyperbilirubin is a rapid, reversible configurational photoisomerisation of the albumin bound lipophilic chromophore to a water soluble form, excretable into the gut.
1.4.7.2. **Plasma formation.**

The application of a large amount of photons in a very short time period results in an intense local electric field. This sudden rise in electrical energy generates a plasma. A plasma can be produced in a variety of ways, the most commonly is dielectric breakdown, whereby large numbers of electrons are released as the conductivity of the material is altered as a result of the intense electric field. This energy is then transferred in a localised manor to the surrounding tissue. Resulting in a small area of vaporised tissue. The rapid expansion of the vaporised material generates an intense shock wave. As the shock wave expands outwards, tissue disruption and ejection of material may result. This mechanism is utilised in the treatment of thickened posterior capsule membranes, which are commonplace following cataract operations. Typically, two infrared beams of subthreshold energies are focused to the spot on the posterior capsule of the lens where membrane disruption is desired. Individually each beams’ power is insufficient to destroy the tissue, however, the two beams together produce a photoplasma that disrupts the tissue\(^{27}\).

The penetration, absorption and scattering of laser energy within tissue depends upon the wavelength of laser light.

**Carbon dioxide laser radiation**- The wavelength of light generated by the carbon dioxide laser (10.6μm) is readily absorbed by water, and since the superficial layers of tissue are composed of more than 50% water, the intensity of laser radiation is reduced significantly within several hundred microns. This feature limits the application of carbon dioxide lasers to the bonding of thin walled vessels in microsurgery.

**Argon laser radiation**- Penetration of the argon laser is considerably higher than that of the carbon dioxide laser, and the coagulation depth is significantly higher. However, because the argon laser operates at wavelengths in the range 488-514nm, the laser light is absorbed preferentially by haemoglobin in the blood, causing
coagulation at areas where it is not necessarily required. Generally argon laser applications are limited to situations where blood concentrations are minimal.

**Nd:YAG laser radiation**- Nd:YAG lasers emitting light at a wavelength of 1.064μm have a greater penetration than both carbon dioxide and argon lasers due to the reduced absorption coefficients of Nd:YAG laser wavelengths in soft tissue. The increased depth of penetration leads to an effect known as redistributive scattering, caused by numerous reflections, diffraction’s and scattering throughout the tissue.

1.5. **LASER ASSISTED VASCULAR ANASTOMOSIS.**

Interest into laser assisted vascular anastomosis developed following studies into the fusion of vascular tissue by electrocoagulation [28]. The application of heat to weld soft tissue is not new. However due to problems associated with the containment and controlling of thermal coagulation, further research using electrocoagulative techniques came to an end. Laser bonding of tissue is thought to involve thermally induced changes within the tissue structure, the changes and the effects these changes have in the bonding is tissue is unknown. The thermal energy generated by the laser induce protein fusion of the vessel wall, in particular, the fusion of collagen [29,30]. There has been a great deal of debate regarding the temperature at which laser fusion occurs. Limited information has been reported concerning the temperature measurements performed during laser welding. However it has been widely reported that the temperature at which bonding occurs is around 65-70°C, this is above the thermal denaturation temperature of collagen. Badeau et al [31] reported a range between 80 and 120°C for carbon dioxide welding. Conversely the optimum temperatures for argon laser welding has been reported as 43 to 48°C [32,33]. Different mechanisms have been reported for tissue welding using the carbon dioxide laser and argon laser. When using the later, rather than denaturing collagen within the blood vessel, it has been proposed that the lower temperatures result in a physio-chemical bond by producing a cross linking of collagen. These beliefs have been reinforced by the work of Schober [34] and White [35].
In 1979 Jain and Gorisch \[^{[19]}\] published a report on the use of Nd:YAG laser radiation to repair cut veins and arteries in rabbits. Observations led Jain to believe that the laser caused heating of the vessel wall, resulting in coagulation of collagen at temperatures in the region 42-65°C (the thermal denaturation temperature of proteins). Expansion to these preliminary investigations soon led to sutureless anastomosis of microvessels \[^{[36]}\]. Three different wavelengths of light have been utilised for the laser bonding of tissue, the carbon dioxide laser \[^{[37]}\], the Argon laser \[^{[35]}\] and the Nd:YAG laser \[^{[36]}\]. Each laser has its own characteristic optical properties in tissue specific to its emission wavelength.

The primary objective of laser welding for use in clinical procedures is that it demonstrates improved patency rates, shorter anastomotic times, a better quality of microvascular anastomosis, and to enable fusion of vessels that cause problems when conventional suturing techniques are employed. Original investigations were performed using blood vessels with diameters less than 1mm. The majority of this work utilised the carbon dioxide laser \[^{[31,37,38]}\] with the exception of Jain \[^{[19]}\] and Godlewski \[^{[39,40]}\] who used the Nd:YAG and Argon lasers respectively.

In contrast to the original method for thermal bonding of blood vessels, laser welding is a non contact technique, and reduces the extent of thermal damage. The research field was extended when investigations into laser bonding of larger calibre vessels using the carbon dioxide laser were undertaken by Okade \[^{[41]}\], Ashworth \[^{[42]}\] and Frazier \[^{[43]}\], although the carbon dioxide laser was known to have limited penetration. Consequently White \[^{[33]}\] and Enynia \[^{[44]}\] employed Argon and Nd:YAG laser light.

Comparisons between investigations using different wavelengths are difficult because of problems associated with vessel preparation prior to anastomosis and doubts over the energy levels required for tissue fusion. The important variables involved in the assessment of the anastomosis are summarised in table 1.2.
Chapter One: Introduction.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Alternative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of anastomosis</td>
<td>End-to-end, end-to-side, side-to-side</td>
</tr>
<tr>
<td>Method of alignment</td>
<td>No of stay sutures, stents</td>
</tr>
<tr>
<td>Laser parameters</td>
<td>λ, Power density, Spot size, Irradiation duration</td>
</tr>
<tr>
<td>Time for irradiation</td>
<td>A glowing of the tissue, ‘the end-point’</td>
</tr>
</tbody>
</table>

Table 1.2. Important variables in the assessment of studies on laser assisted vascular anastomosis

Laser welding involves the apposition of the two vessel edges by stay sutures or even vascular clamps. The imperative feature is that the vessels are as close to one another as possible. The treatment of the vessel edges before lasing varies depending upon two different hypotheses. The original study by Jain and Gorisch [19] employed vessels in their original native state (blooded), whereas Neblett et al [37] removed all blood before irradiating, as it was thought to alleviate problems associated with weak secondary bond formation. It has been reported [45] that a coating of blood over the vessel can adversely affect the welding process, by absorbing unpredictable amounts of laser radiation.

Vessels are coapted together by applying tension to the stay sutures and then are irradiated in a continuous manner, until exposure causes a slight blanching and contraction of the tissue. This section reviews the existing literature on laser welding.

1.5.1. **Laser assisted vascular anastomosis using the Nd:YAG laser.**

The Nd:YAG laser was the first laser to be employed for laser welding. Arteriotomies [19] were performed on rabbit microvessels, using laser, with an optical fibre arrangement and a microscope. Further studies by the same author [46,36]
involved the end-to-end and end-to-side anastomosis of rat carotid arteries. A six month success rate of 90% was reported. The anastomoses withstood intravascular blood pressures up to 3000mmHg, and microscopic examination revealed that the endothelial layer was regenerated across the repaired vessel within 2-3 days. The principle difficulty associated with these investigations was the inability to determine the end-point for irradiation. Furthermore, there was no histological evidence to indicate a degree of vessel trauma, and no control sutured group to assess the potential of Nd:YAG lasers for vascular welding. Regardless of these problems Jain performed five sutureless extra-intracranial bypass operations on human patients. A power of 18 watts was delivered by a 600μm quartz fibre system connected to a handpiece containing a 50mm lens (0.3mm spot obtained). The irradiation duration was a single 0.1 second application. The laser beam was advanced from one ‘spot weld’ to the next manually. All five patients exhibited satisfactory anastomoses in the nine months following surgery, with no evidence of aneurysm formation. The advantage of this technique was the speed of anastomoses (5 minutes compared to 15 minutes for conventional suturing).

A more recent study involved the anastomosis of rat carotid arteries using laser without stay sutures. Earlier studies by the same author determined that the optimum bonding conditions were a power of 20 watts applied for 100 milliseconds. An intraluminal splint made from water soluble PVA was used for precise vessel coaptation (the splint dissolved on the recirculation of blood). A 92% patency rate was achieved 24 hours after surgery and all vessels remained patent until the 30th postoperative day. Tensile strength investigations immediately following surgery, and one week later, indicated that the anastomoses generated by the laser were significantly better than those achieved by the usual sutured techniques. Niijima’s studies came to the conclusion that the use of the PVA splint improved tissue fusion, with minimum foreign body reaction.

Conversely, in a preliminary comparative study into the ability of Argon and Nd:YAG lasers for vascular anastomoses, the mechanical strength and degree of vessel
constriction in anastomoses generated by the two lasers were investigated. Four hundred porcine coronary anastomoses were prepared in vitro. Vessel constriction was measured by a pressure flow rate method, and bursting pressure by saline infusion. No major difference between the two lasers was evident so far as bursting pressures were concerned, although they were both significantly weaker than the control group of sutured anastomoses. The degree of vessel constriction was greater for vessels repaired by the Nd:YAG laser. (see table 1.3).

<table>
<thead>
<tr>
<th>Laser</th>
<th>Burst pressure/mmHg</th>
<th>Flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon</td>
<td>280±22</td>
<td>85±3</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>273±30</td>
<td>64±7</td>
</tr>
<tr>
<td>Control</td>
<td>&gt;500</td>
<td>93±2</td>
</tr>
</tbody>
</table>

Table 1.3. A comparison of the best results produced for each laser for laser anastomoses of coronary arteries in vitro.[50]

1.5.2. **Laser assisted vascular anastomosis using the Argon laser.**

The Argon laser has a greater penetration depth in tissue than the carbon dioxide laser, and has been used for the anastomosis of larger diameter vessels. Gomes et al performed three types of vascular anastomoses in twenty four mongrel dogs, saphenous vein internal carotid artery bypass graft, left mammary artery/left anterior descending coronary artery bypass, and veno-venous anastomoses. In each group, a laser power of 0.8 to 1.5 watts was used for total irradiation durations ranging from 90 to 300 seconds. The average bursting pressure of the laser anastomosis was 730mmHg immediately after irradiation. This figure increased to 2500mmHg thirty days following surgery. The results were satisfactory, however assessment of the quality of anastomosis was difficult because only a few samples were repaired. Pribil
performed 30 end-to-end anastomosis on rat carotid arteries using laser with two supporting stay sutures. The success rate immediately following surgery was 100%, however when the repairs were reviewed one month later, the patency had dropped. Fifty five percent of the anastomoses showed signs of stenosis and 77% developed aneurysms. An interesting point of this study, was that Pribil reported a visual endpoint.

More recently, Vance et al repaired longitudinal slits in rats abdominal aorta. Forty two animals underwent laser welding, while 15 animals were repaired by conventional sutured anastomosis. An eight day patency rate of 100% was observed in the sutured repair group compared to a 60% rate for the vessels subject to laser repair. However, no further failures were recorded in either group if the animals survived longer than 8 days. Bursting pressure investigations on both groups of animals gave bursting pressures consistently higher than 500mmHg. Vessels subjected to laser repair revealed a 29% pseudo aneurysm formation (greater than the sutured group), however, the conventional sutured group exhibited a greater foreign body reaction. With the aid of a chromophore, a study was undertaken to assess the relative merits of laser and sutured anastomosis. Twenty canine bilateral femorofemoral bypass grafts were fashioned to bypass the diseased common femoral artery. In each case, one graft underwent conventional sutured anastomosis, while the other utilised laser and an appropriate chromophore. A power density of 6 watt/cm² was employed with a spot diameter of 2mm, a series of eight ‘spot welds’ were fashioned per anastomoses with an exposure time of 30 seconds per spot. On average, the operating time for the laser anastomosed group was greater than the conventional sutured group. Brooks' research came to the conclusion that no definite advantages resulted from the laser anastomosis techniques.

1.5.3. Laser assisted vascular anastomosis using the carbon dioxide laser.

The carbon dioxide laser has very little penetration in biological tissue. Investigations involving carbon dioxide lasers have been limited to the microvascular anastomosis of animal blood vessels. Morris and Carter used a milliwatt carbon dioxide laser
to attempt welding of vessels opposed by stay sutures. The technique was taken up by others, some of whom claimed that the technique was faster and easier than conventional techniques. Quigley \cite{57} prepared anastomoses of rat femoral arteries with laser and the aid of several stay sutures, additionally a comparative control sutured group was used. This initial study focussed on detailed histological investigations. Immediately after laser irradiation there was a loss of endothelium and platelet aggregation on the inner surface of the weld. Coagulation necrosis was observed in the medial layer of the artery, and collagen necrosis in the adventitial layer. Three days following anastomosis, platelet aggregation increased and the area of medial necrosis was easily noticeable when compared to the surrounding media where smooth muscle cells were growing. Endothelium cell growth and platelet aggregate decline commenced at one week following surgery, furthermore, smooth muscle cells continued to proliferate into areas adjacent to the necrotic medial layer. Importantly, a definite region of foreign body reaction was noticed around the stay sutures. Three weeks after surgery, endothelial cells had expanded over a thickened intima and elastic fibres were present in the media. This study concluded that medial necrosis was greater in the laser anastomosed group, while the extent of endothelial loss, reendothelialisation time, and degree of platelet and fibrin deposition were almost identical between the two. In a later study by the same research group \cite{58}, 113 rats underwent laser assisted femoral artery anastomosis, with an additional control group of 67 repaired by sutured anastomosis. Three months following surgery the anastomoses were assessed for aneurysm formation, a 30\% rate was reported for the laser welded groups in comparison to no aneurysm formation for the sutured group. Flemming et al \cite{55} used a milliwatt CO$_2$ laser to conduct a series of anastomoses of rat arteries and veins. Three stay sutures were inserted prior to lasing to approximate the vessel edges. The vessels anastomosed by a laser welding technique were compared with a group repaired by conventional suturing, for patency, speed, and aneurysm formation. A total of 265 vessels, with diameters ranging from 0.6 to 2mm were anastomosed using laser, with 119 arteries and veins being anastomosed using conventional techniques. On average, sutured repairs were complete within
approximately 9 minutes, compared to 6 minutes for the laser welded groups. Success rate for ‘first time successful’ laser anastomosed vessels were comparable to those repaired using suturing (98.8% compared to 98.0%). However, there were a number of vessels that required further welding after the removal of the supporting clamp. The patency rates of this group were significantly lower than the initial successful anastomoses (71.1% compared to 98.8%). Aneurysm formation was evident in both experimental groups, the majority were only small (<1mm), although eight aneurysms occurred in the laser group that were greater than 2mm in diameter. The incidence of aneurysm formation in the laser group was twice that of the conventional sutured vessels (38.8% compared to 14.3%). This study came to the conclusion that although laser welding was governed by a large number of factors, it offered a high success rate and that further work was required to optimise laser parameters and improve results.

Okada et al [41] performed in vivo anastomoses on sixty five dogs. All three different anastomotic arrangements were performed for operation on femoral/carotid arteries and veins. The diameter of these vessels ranged from 2-10mm. A laser power of 20-40 milliwatts was used with an irradiation duration of 60-120 seconds. The anastomoses were studied from 6 hours to 2.5 months after surgery. During this period, there were no deaths as a result of failures of the anastomoses. The quality of the anastomoses was assessed by angiographic studies, pressure tolerance tests, tensile strength tests and histological examination. No haemorrhages were observed with the repaired vessels even when the intravascular pressure approached 300mmHg. Tensile strength tests concluded that there was no significant difference between conventional suture methods and laser anastomosis. Histological examination revealed that proliferation of fibroblasts and collagen fibres on the anastomotic line were observed. Healing around the anastomoses was good, but not fully understood. It was stipulated that the collagen fibres are changes from a gel to a solid like paste as a result of laser heating.
1.5.4. **Chromophore assisted laser vascular anastomosis.**

A chromophore is the colour bearing component of a material. Such compounds can be used in laser welding to obtain preferential absorption of the laser at the site of the anastomosis. Application of a chromophore minimises the spread of thermal damage and allows a reduction in the incident laser energy required for consistent bonding. Investigations into chromophore assisted laser anastomosis are a relatively new area of research, and studies have been limited to Argon and Nd:YAG laser applications.

Vance [59] used an infrared radiation thermometer to measure the temperature profile of Argon laser irradiated tissue in situations with no chromophore, chromophore on the external surface of the vessel and chromophore on the internal surface. The rate of temperature rise was greatest with the chromophore applied to the external surface and irradiated with a power of 0.3 watts, less with the chromophore on the internal surface at the same power, and least with no chromophore at a power of 0.5 watts. This investigation demonstrated the preferential absorption of laser power by the chromophore, and the reduced power density reaching the internal surface of the vessels. At \( \approx 65-70^\circ C \), the established end-point for laser welding, there was a temperature difference of \( 17^\circ C \) between the external and internal surfaces with no chromophore applied. With chromophore on the external surface, the temperature difference was 20\(^\circ C\), but there was no temperature difference when the chromophore was placed on the internal surface. Using a surgical stain, Basic fuchsin [59], chromophore assisted laser welding was assessed. End-to-end anastomosis of pigs coronary arteries were prepared by laser. Application of the chromophore allowed a large reduction in the incident laser power. Anastomosis with chromophore, irradiating using a power of 0.3-0.5 watts with only one supportive stay suture, generated a bond with a bursting pressure in the range 90-310mmHg. Whereas without chromophore a laser power of 1 watt was required to create welds with a bursting pressure of 90-350mmHg. Further investigations using the same chromophore [53], focussed on the *in vivo* repair of larger diameter blood vessels.
Rather than fashioning anastomoses, a longitudinal incision in a rat abdominal aorta was repaired. The results from the group of vessels repaired by laser and chromophore showed higher first eight day failure rates (40%) when compared to sutured control groups. However, it was found that if animals survived longer than eight days, no future failures of the repaired vessels would occur. Bursting pressure results revealed that all of the repairs withstood pressures exceeding 300mmHg (the normal physiological range). Chuck [60] performed a series of in vitro longitudinal arteriotomies using Argon laser with and without the chromophore Fluorescein isothiocyanate. The degree of thermal damage was measured histologically. Bursting pressure studies revealed that there was little difference between the strengths of the vessels repaired with or without chromophore (164mmHg with chromophore, 147mmHg without). Histology results concluded that aortotomies prepared with the addition of chromophore exhibited decreased collateral thermal damage.

Few reports exist on the use of chromophores with the Nd:YAG laser. Brooks [61] studied a number of infrared absorbing dyes, including several developed by the University of Leeds. Fen 6, a ferric-nitroso complex is a commercially available dye. This particular chromophore was used in combination with a Nd:YAG laser for the in vitro preparation of longitudinal arteriotomies in porcine coronary arteries. The study was undertaken to assess the relative merits of the Argon and Nd:YAG lasers, and to investigate the use of chromophores as potential aids for laser welding. Comparison of the optimum results for each category of weld (table 1.4) revealed that Argon welds were significantly stronger than Nd:YAG welds, without chromophore. However, this difference was not evident when chromophore was used with each laser. The use of chromophore increased the weld strength, the most significant difference was noticeable for the Nd:YAG laser repairs.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Power density</th>
<th>Irradiation duration</th>
<th>Mean bursting pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon</td>
<td>40W/cm²</td>
<td>20-60 sec</td>
<td>249mmHg</td>
</tr>
<tr>
<td>Argon+CR</td>
<td>4.6W/cm²</td>
<td>60 sec</td>
<td>282mmHg</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>197W/cm²</td>
<td>45 sec</td>
<td>162mmHg</td>
</tr>
<tr>
<td>Nd:YAG+CR</td>
<td>105W/cm²</td>
<td>60 sec</td>
<td>271mmHg</td>
</tr>
</tbody>
</table>

+CR = With chromophore.

Table 1.4. A comparison of the optimum results produced for each category of laser welded arteriotomy repair \[61\].

1.5.5. **The use of biological sealants to improve surgical results of laser assisted vascular anastomosis.**

The use of biological sealants in laser welding is an area of research in its infancy. Fibrin based sealants have regularly been used in conventional anastomosis \[16,17,18\] (see section 1.2 ). Poppas et al \[62\] investigated the use of a tuneable krypton ion laser (λ=532nm) to perform a patch graft urethroplasty in 24 adult male dogs. Two different experimental arrangements were assessed. Twelve repairs were completed using conventional suturing techniques and a further twelve repairs were completed using the laser and a protein solder combined with the chromophore, Fluorescein. Throughout the experiments a laser power of 0.7 watts was used with short irradiation bursts of 0.2 seconds and a spot size of 400μm, the repair was fashioned as a series of ‘spot welds’. The calculated power density per pulse was 5.6W/cm². Immediately following repair, six animals from each group underwent intraluminal bursting pressure testing. The average bursting strength for the suture group was 10.2±5.4mmHg, compared to 39.5±15.6mmHg for the laser plus solder repair. The
remaining six animals in each group were observed over a six week period. Six weeks after repair the animals were sacrificed and sections of the urethra containing the repair were removed and submitted for histological investigation. The results from these studies revealed that the suture repair group exhibited greater fibroblastic response and showed inflammation with giant cell formation analogous with reaction to suture material. Conversely, the laser repair group showed much less inflammation with no giant cell reaction. The operating time was 42% faster for the laser/protein glue repair group.
1.6. **Spectroscopy of excited states.**

Photochemistry involves the use of light to produce electronically excited states of molecules. The electronically excited molecule is energetically unstable with respect to the ground state. If the molecule does not undergo any chemical rearrangement, it will find a way of losing the energy to return to the ground state. There are a number of different ways in which the molecule can lose its excitation energy. The most favoured paths depend on the type of molecule and the nature of the electronic states involved. The different paths are classified according to three categories.

**Radiative processes.** Involving the emission of electromagnetic radiation by the excited molecule as it returns to the ground state.

**Non radiative processes.** In this case, the population of the electronically excited state are transferred into others without the emission of radiation.

**Quenching processes.** These processes involve the transfer of energy from the electronically excited molecule to other molecules that are in collision. However in addition, non-radiative quenching can occur. Dipole-dipole long range transfer occurs due to coupling of transition dipoles.

It is interesting to probe the fates of electronically excited states. The probe may be a second light source, measuring changes in the transmittance of a sample as a function of time. Investigations into the decay of the excited states of molecules falls into two categories, time resolved and steady state techniques. In this section, optical methods for monitoring the production and decay of excited state species will be presented.

1.6.1. **Steady state absorption techniques.**

The ground state absorption spectra is used to investigate the light absorbing properties of the ground state of a molecule. Typical UV/Visible ground state spectrophotometers consist of an excitation source, in the form of a tungsten lamp for use in the visible region, and a deuterium lamp for the ultra violet region. The wavelength where the absorption is to be measured is selected from either excitation lamp using a monochromator. If the sample is present in dilute solution, it is
necessary to correct for absorption by the solvent and the cuvette, especially in the ultra violet region. Typically, in modern spectrophotometers, a double beam arrangement is used, so that the incident beam is split into two, and passes through two optically matched cuvettes. One contains the sample and the other contains the pure solvent. The double beam arrangement also corrects for possible variations in lamp intensity as different wavelength are scanned. The light transmitted by the sample and the reference is detected by a photomultiplier.

1.6.2. Steady state emission techniques.
Measurements of the steady state emission consist of a suitable continuum source of UV/Visible radiation, typically a xenon arc lamp, to generate a steady state population of excited states. This radiation is passed through a monochromator to select an excitation wavelength. The sample under investigation is contained in a 1 cm$^2$ quartz cuvette. It absorbs the excitation radiation and emits fluorescence. The emitted radiation is at right angles to the excitation beam and is focused onto the entrance slit of a further monochromator, which disperses the emission radiation and a wavelength is selected to be detected by a photomultiplier. Because two monochromators are present, two different types of spectra can be measured. A fluorescence emission spectrum is measured by setting the excitation monochromator to a wavelength known to be absorbed by the sample. The emission monochromator is then scanned over the wavelength region of the emission. A fluorescence excitation spectrum is measured by setting the second monochromator to a fixed wavelength, typically near the emission maximum of the sample. The excitation monochromator is then scanned to determine the wavelength at which sample absorption leads to emission at the selected wavelength. The fluorescence excitation spectrum yields information that is complementary to, but not necessarily the same as the absorption spectrum of the sample. When investigating dilute solutions, fluorescence is measured at right angles to the axis of the incident exciting radiation, in order to reduce the amount of scattered light emanating from the excitation source.
A similar experimental arrangement can be used to measure phosphorescence spectra. Phosphorescence is observed to occur from the lowest excited triplet state, T<sub>1</sub>, and it only occurs with a high efficiency in rigid media. Because the radiative transition from T<sub>1</sub> to S<sub>0</sub> is spin-forbidden, the radiative lifetimes are very long. Phosphorescence usually occurs after a longer time in relation to fluorescence. Consequently choppers are placed between the excitation source and sample, and the sample and photomultiplier, which are modulated out of phase with one another, so that no light can reach the photomultiplier when the sample is being excited. This ensures that the long lived phosphorescence can be detected, but the short lived fluorescence is not.

1.6.3. **Micro- and nano-second flash photolysis.**

Flash photolysis has an advantage that it does not require the excited states to be emissive. It can be used to follow the formation and decay of excited states that do not emit. Flash photolytic techniques involve subjecting a solution containing the sample of interest to a short intense burst of light. Originally flashlamps were used to generate the pulse of radiation\textsuperscript{[63]}, however, more recently Q-switched lasers are used\textsuperscript{[64]}. The short burst of incident light results in the generation of a high concentration of excited states. The decay of these excited states is monitored using a second light source, usually a xenon arc lamp is used, however it is possible to use continuous wave lasers. The decay of the excited states is monitored as the difference in absorption between the system before excitation, and the system at a particular time t after the onset of excitation. Photomultipliers and transient digitisers or even digital oscilloscopes can be used to monitor real time events, if the excited state decay is in the micro- or nano-second timescale regime.

1.6.4. **Single photon timing.**

Single photon timing is the most widely used method for determining emission lifetimes. Excitation is provided by a pulsed flashlamp or a laser. The flashlamps are generally filled with either nitrogen or hydrogen depending on the required excitation
wavelength. The pulse width of the excitation source can be varied by altering the gas pressure, repetition rate, discharge voltage and electrode spark gap, with the shortest attainable pulse being about 1 ns. However there is competition between the lamp intensity and pulse width, consequently it is necessary to vary these two conditions depending on the emission quantum yield and lifetime to be measured. In comparison to flash photolytic techniques, where a single pulse of exciting light leads to the detection of one decay profile, single photon counting may use 100 pulses of exciting light leading to the detection of one emission photon. A typical single photon counting apparatus is illustrated in figure 1.5. The instrumentation consists of two photomultipliers. The first located near the source, the ‘start’ photomultiplier detects the emission of an excitation pulse and starts a counter. This excitation pulse then excites the sample into an upper excited state. From this point there may be various non-radiative transitions before the final emission state is reached. Emission from this state then occurs, the probability of emission at a particular time is dependant on the population of the emission state, and therefore on the length of time after the excitation pulse resulting in emission. When the sample emits radiation it is detected by a second photomultiplier, the ‘stop’ photomultiplier, this generates a pulse to stop the counter. The time between start and stop pulses is converted to a pulse by a time to amplitude converter (TAC), the amplitude of this pulse is proportional to the time. This pulse is digitised by a multichannel analyser and a memory channel corresponding to this digital value is increased by one. Consequently a histogram is generated where the height of the pulse corresponds to the probability of a photon being emitted by the sample at a given time after excitation, as described earlier, this is related to the population of the emission state and ultimately to its lifetime. Typically the exciting and emitted wavelengths are isolated using monochromators or suitable cut-off filters. For the analysis of an emission decay it is important to collect a large number of start and stop events, usually in the region of 10000 counts in the channel with the highest number of counts. Therefore single photon timing measurements usually require a long sampling time even when using high repetition rate sources, and especially where the sample emits with a low quantum yield or a long lifetime.
Furthermore, when using single photon counting methods it is important to determine a reference signal from the apparatus, this is known as the instrument response function, and reflects the response of the instrument to the actions described above. It is dependent on the pulse width of the exciting source, in addition to the response times of the photomultipliers and electronics. For short pulse widths, the later characteristics become important considerations. When fitting kinetic models of the data it is necessary to convolute the instrument response function with the measured emission decay.

Figure 1.5: Schematic diagram of a single photon timing apparatus.
Chapter One: Introduction.

1.7. EXPERIMENTAL.

1.7.1. Ground state measurements.
All ground state absorbance spectra were recorded using a Phillips PU-8800 UV/Visible spectrophotometer. Emission spectra were recorded using a Spex Fluoromax™ fluorimeter. Both instruments were interfaced to IBM compatible computers, and the software controlling these instruments was either purchased with the instrument or written specially by members of the Photochemistry research group at Loughborough.

1.7.2. Single photon timing measurements.
The Single photon timing measurements were performed in the laboratory of Prof. D. Oelkrug at the University of Tubingen, Germany. The measuring apparatus consisted of a Spex Fluorolog 112 fluorimeter, equipped with a Peltier-cooled Hamamatsu R928 photomultiplier. Excitation was provided by a PRA 510B flash lamp, filled with a 1:1 mixture of Nitrogen and Hydrogen gas.

The electronic equipment used for controlling and acquiring the single photon timing data consisted of the following ORTEC components. A MCA 6220 multi channel analyser, Model 454 amplifier, a 100MHz discriminator (Model 436), a constant fraction discriminator (Model 463) and a time calibrator (Model 462).

1.7.3. Nanosecond laser flash photolysis.
1.7.3.1. Collection of data.
A Q-switched Nd:YAG laser JK 2000 (JK lasers, now Lumonics) was used during the flash photolysis studies. The wavelengths of light available from this laser for use in photochemical experiments are the second (532nm), third (354nm), and fourth (266nm) harmonic of the Nd:YAG laser fundamental wavelength at 1064nm. However, only the 532nm excitation wavelength was used during these experiments. A 300W Xenon arc lamp (Oriel 6258 Ozone free) was used as the analysing source. Light detection is achieved by an f2.4 grating monochromator (Applied photophysics
ltd) and a R928 side window photomultiplier tube (Hamamatsu). A high voltage power supply (Applied Photophysics Photomultiplier Supply) is used to provide the accelerating voltage to the photomultiplier. The output voltage from the photomultiplier tube is connected to a transient digitiser (7612D Tektronix) with a differential comparator plug-in (7A13 Tektronix). The digitiser is interfaced to an IBM compatible computer, through a general purpose interface bus (GPIB). This arrangement allows setting of the required parameters during experimentation and enables passing of data from the detection system to the computer for later analysis. Two shutters are used in this apparatus, one placed between the arc lamp and the sample, and the other between the laser and the sample. This arrangement allows irradiation of the sample by non, either or both light sources. The shutter control is achieved through the computer via a digital/analogue/analogue/digital card (DT 2808 Data Translation).

A schematic diagram of the nano-second flash photolysis apparatus is shown in figure 1.6 [65]. Control and timing of the system is achieved by a quartz oscillator and a series of analogue delay modules.
The flash photolysis system works as follows;
1. The computer receives a trigger.
2. The computer via the GPIB interface instructs the digitiser to arm its timebase and to digitise a signal when it receives another trigger. The computer then waits for the digitiser to acknowledge that the digitising sequence has completed.
3. The particular shutters are activated, the two shutters placed between the arc lamp and sample, and laser and sample allow recording of the topline (no shutters open), baseline (arc lamp shutter open), transient absorption (both open) and emission (laser shutter open).

The firing of the JK laser triggers the digitiser. This is done by reflecting part of the laser beam off a glass plate into a fibre optic, which is incident upon a photodiode. When the digitiser receives the trigger, the signal at the input is digitised. When this
process is complete, the information is transferred to the computer, where it is shown on the screen and saved to memory for later analysis.

1.7.3.2. Data Analysis.

After recording the baseline, topline, transient absorption and emission traces using the flash photolysis apparatus, they are used to calculate the required data by:

1. The data corresponding to the corrected transmission change is produced by subtracting the emission trace from the transient absorption trace. (It is important to note that this calculation is only possible if the emission trace stays on the screen, and does not overload the photomultiplier).

2. The range of the screen is determined by subtracting the topline from the baseline. This procedure makes sure that the calculated transmission change is independent of the position of the baseline and topline.

\[
\Delta T = 1 - \frac{\text{transmission}_c}{\text{baseline}_c} \tag{1.5}
\]

the subscripts \(c\) are the corrected baseline and transmission by considering the topline and emission. The value of interest in solution flash photolysis is the absorbance change, this is calculated from Beers law.

\[
\Delta A = \log_{10} \left( \frac{1}{1 - \Delta T} \right) \tag{1.6}
\]
2. **DETERMINATION OF THE OPTICAL PROPERTIES OF PORCINE VASCULAR MATERIAL**

2.1. **Introduction.**

As the use of lasers becomes increasingly common in numerous medical applications there is a need to understand how this radiation propagates in tissue. Knowledge of the optical coefficients of tissue are invaluable for the development of therapeutic techniques such as laser surgery and photodynamic therapy.

Laser assisted vascular anastomosis involves using laser radiation to thermally denature blood vessel proteins such as collagen. Control of the tissue temperature is particularly important for the success of the technique, since if the temperature of the blood vessel exceeds 200°C, tissue carbonisation ensues and blood vessels are damaged irreversibly.

Calculations of the expectations of the amount of heat generated within laser irradiated tissues have been based simply on Beer’s law of absorption of the laser beam, which neglects scatter. It is assumed that the resulting heat decreases with penetration depth below the irradiated surface as if the laser was propagating as a collimated beam. However, at the majority of laser wavelengths used in medical applications, scattering affects the distribution of light within the tissue, and laser radiation diffuses sideways. This results in an extension of the heat source beyond that expected for a collimated laser beam.

In a review article by Cheong, the optical properties of a variety of tissue samples measured using a number of different techniques are presented. The relative merits of each technique are discussed. Comparisons between measurements of the optical properties of different tissue samples are difficult, and efforts to determine suitable optical properties are extremely complicated because of:

- The differences in the physiological condition of the tissue.
- The degree of hydration of the tissue sample.
- Whether the measurement is made in vivo or in vitro.
The most commonly used method for determining the absorption and scattering coefficients of tissue is the Kubelka-Munk two flux theory \(^{[20,68]}\). The advantage of this approach is that the optical coefficients can be calculated from diffuse transmission and diffuse reflection measurements. These measurements can be made using an absorption spectrophotometer with an integrating sphere attachment. The use of integrating spheres for measuring diffuse reflectance and transmission have been widely studied, the theory behind the technique is well documented \(^{[69,70,71]}\). The relationship between diffuse transmission \(T_d\), diffuse reflectance \(R_d\) and the Kubelka-Munk absorption and scattering coefficients are \(^{[20]}\).

\[
\begin{align*}
    a &= \left(\frac{S+K}{S}\right) = \frac{1}{2} \left( \frac{1}{R_\infty} + R_\infty \right) \quad (2.1) \\
    b &= \sqrt{a^2 - 1} \quad (2.2) \\
    S_{KM} &= \frac{1}{bd} \left( \sinh^{-1} \frac{b}{T} - \sinh^{-1} b \right) \quad (2.3) \\
    A_{KM} &= (a - 1)S_{KM} \quad (2.4)
\end{align*}
\]

where

- \(T\) is the fraction of incident light transmitted by the sample
- \(R_\infty\) is the reflectance of a layer so thick that any further increase in thickness fails to change the reflectance
- \(S_{KM}\) is the Kubelka Munk scattering coefficient (cm\(^{-1}\))
- \(A_{KM}\) is the Kubelka Munk absorption coefficient (cm\(^{-1}\))
- \(d\) is the thickness of the sample (cm)
and a and b are parameters as defined by equations (2.1) and (2.2).

However, there are disadvantages of the Kubelka-Munk technique. Firstly, the results are limited to irradiation with diffuse uniform light, and secondly they are only applicable to measurements on perfectly diffuse reflectors.

2.1.1. **For dominant absorption.**
At wavelengths where absorption by tissue is very strong, particularly in the ultra violet region and in the far infra red, the scattering of laser light within the tissue is negligible and the absorption coefficient determines the penetration depth of the laser, and the light does not spread outside the laser irradiated region. In this instance, the attenuation of light in direction z within tissue can be described by Beer's law, thus.

$$I_C = I_0 \exp[-(A + S)]z \approx I_0 \exp(-Az) \quad \text{when} \quad A \gg S \quad (2.5)$$

where

- $I_0$ is the incident laser irradiance (W/cm$^2$)
- $I_C$ is the laser irradiance at penetration depth z (W/cm$^2$)
- $A$ is the absorption coefficient (cm$^{-1}$)
- $S$ is the scattering coefficient (cm$^{-1}$)

At laser wavelengths when absorption dominates scattering processes, the above equation can be used in equations for the determination of the temperature rises in laser irradiated tissue.

2.1.2. **For significant scattering.**
In cases where the light scattering in tissue is significant, the amount of scattered light from the collimated laser beam causes a significant diffuse component of the irradiance at a point in the tissue. The rate of heat generation in these situations is given by\textsuperscript{[72]},

$$Q(r) = A(r) \int L(r,\omega) d\omega \quad (2.6)$$

where $Q(r)$ is the heat source (W/cm$^3$)
A is the absorption coefficient (cm\(^{-1}\))

\(L\) is the radiance (W/cm\(^2\)-sr\(^{-1}\)) at position \(r\) in the \(s\) direction

\(\omega\) is a solid angle, and the local fluence rate \(\mathcal{G}\) is defined as \(\int_{4\pi} L_{(r,s)}d\omega\) (Wcm\(^{-2}\)).

The heat source \(Q\) is proportional to the absorption coefficient and the irradiance. Estimations of the rate of heat generation demand the accurate determination of both collimated and diffuse components of the radiance. Beer's law can be used for describing an absorption coefficient for collimated light with penetration depth, if it is possible to measure through the tissue sample. However, the determination of the diffuse radiance is considerably more difficult. When scattering is significant, it is necessary to determine three optical coefficients: the absorption coefficient for collimated light \(A\), the scattering coefficient \(S\) and the anisotropy factor of scatter, \(g\).

To determine all three coefficients, it is necessary to measure the total diffuse reflection and transmission under diffuse irradiation and in addition the transmission of collimated light through a tissue sample. The equations relating Kubelka-Munk coefficients to the absorption, scattering and anisotropy coefficients have been given by several authors\(^{[73,74,75]}\) including Welch as\(^{[76]}\).

\[A = \frac{A_{KM}}{2}\] (2.7)

\[T_c = \exp[-(A + S)d]\] (2.8)

\[S = -\frac{1}{d}\ln T_c - \frac{A_{KM}}{2}\] (2.9)

\[S_{eff} = S(1-g)\] (2.10)

\[S_{KM} = \frac{3}{4}S_{eff} - \frac{1}{4}A\] (2.11)

\[g = 1 - \left(\frac{A_{KM} + 8S_{KM}}{6S}\right)\] (2.12)
where
g is the anisotropy factor
\( T_c \) is the collimated transmission.

The main difficulty with this approach is to determine a correct value for the collimated transmission, \( T_c \), because of the mismatch of the sample/phase boundary. The collimated transmission is very small and it is difficult to measure correctly, although Welch et al. attempted to measure its magnitude by incorporating the sample and the whole of the detection system in a water tank, so as to avoid the problem associated with a mismatch in the refractive indices.

Because of the problems associated with the Kubelka-Munk approach outlined above, a new method is presented that can be used to determine optical properties of tissue from laterally resolved reflection measurements under point irradiation. Oelkrug and Brun have presented the principle of laterally resolved reflection measurements in several papers. The technique has been used to measure the absorption and scattering coefficients of various homogeneous systems including typical substrates for thin layer chromatography such as alumina, silica and cellulose. The measuring geometry is shown in figure 2.1.
Figure 2.1: Geometry to measure back-scattered light as a function of the distance \( r \) from the axis of the incident beam

An area of the surface of the sample is illuminated under normal incidence with a focussed laser beam. The relative radiance \( R(r) \), the ratio of the detected radiance and the incident radiant flux is measured as a function of the distance from the centre of the point of illumination. The shape of the relative radiance curve is affected by the scattering and absorption properties of the sample. The diffusion of photons from the initial point of irradiation is determined by the effective mean path between scatterers, which is defined as:

\[
< r > = [S(1-g)]^1
\]

(2.13)

For samples with low effective scattering coefficients, photons diffuse over a larger area compared to samples with high effective scattering coefficients. As a result, the
relative radiance curve as a function of distance is less steep. When absorption occurs, scattering at longer distances from the point of irradiation is more affected than at shorter distances and the relative radiance curves reach a lower maximum and fall off more steeply. The difference between the refractive index of the sample and the boundary also affects the shape of the relative radiance curve. Because of the occurrence of total internal reflection, rather than photons being transmitted through the sample, they are total internally reflected, and photons diffuse further from the initial point of irradiation. This results in a broader relative radiance curve.

The two major difficulties in the experimental determinations of tissue coefficients are the anisotropy of scattering and internal Fresnel reflection. In homogeneous samples such as microcrystalline silica, the light is scattered evenly in all directions (this is known as isotropic scattering). However, when scattering occurs within tissue, rather than being scattered evenly, light is scattered in the same direction, and only deviates slightly from the collimated transmission. Isotropic scattering is denoted by a g value of 0, and totally forward scattering by a g value of 1. The anisotropy parameter, equal to the average cosine of the scattering angle is commonly equal to 0.8 or higher in animal tissues. In cases were the anisotropy factor is significant, the original scattering coefficient is replaced by an effective scattering coefficient $S_{\text{eff}}$, which takes into account the isotropic scattering, $S_{\text{eff}}=(1-g)S$.

For a semi-infinite medium, the boundary conditions have to be defined. In cases where the refractive index of the tissue matches that of the outside medium, the boundary conditions are simple and there is no reflection of the photon back into the tissue from the outside medium. Duderstadt and Hamilton derived a mathematical expression describing this refractive index matching. Putting $\omega(\tau)$ equal to $L_{\omega(\tau)} d\omega$, they obtained the following expression.

$$\phi_{(0)} - 2D\Omega_n \cdot \nabla \phi_{(0)} = 0$$

(2.14)

where $\phi_{(0)}$ is the fluence rate (W/cm$^3$).
Chapter Two: Determination of the optical properties of porcine vascular tissue.

D is the diffusion constant which is equal to $3[A+(1-g)S]^{-1}$, A and S are the absorption and scattering coefficients respectively ($\text{cm}^{-1}$).

$r$ is a point on the interface of the sample and the surroundings.

$\Omega_n$ is a unit normal vector directed into the tissue.

However, when dealing with tissue, the refractive index of the tissue differs from that of the external medium. Consequently, the boundary conditions must be changed to allow for internal reflection at the surface. For a mismatched boundary, the boundary condition can be written as $^{[81]}$.

$$\phi(0) - 2AD\Omega_n \cdot \nabla \phi(0) = 0 \quad (2.15)$$

where $\phi(0)$, D, $\Omega$ and $r$ are as defined above,

$A$ is related to the internal reflection and can be derived from the Fresnel reflection coefficients as:

$$A = \frac{\left(\frac{2}{1-R_o} - 1 + |\cos \theta_c|^3\right)}{1 - |\cos \theta_c|^2} \quad (2.16)$$

where $\theta_c$ is the critical refractive angle

$R_o$ depends upon the relative refractive index, $n_{rel} = n/n_r$ of the tissue air interface,

$$R_o = \left[\frac{n_{rel}-1}{n_{rel}+1}\right]^2$$

2.1.3. **Monte-Carlo method.**

Monte Carlo simulations have been applied to tissue optics by Groenhuis et al $^{[82]}$ and Wilson $^{[83]}$. The advantage of the Monte Carlo simulation is that it is flexible as regards geometry, phase functions and boundary conditions. The chief disadvantage is that the method is computationally expensive and requires great computer power.
Although faster computers will help, the limiting factor is that the accuracy of the simulation only increases with the square root of the number of photons used for the simulation.

The basic steps of the Monte Carlo simulation are:
1. Photon generation
2. Pathway generation
3. Scatter or absorption
4. Detection.

In a recently published paper, Oelkrug and Brun \cite{78} describe the fundamental steps of the Monte Carlo simulation that were used to obtain radial reflectance curves for comparison with experimental measurements of the laterally resolved reflectance in samples of porcine aorta.

The work detailed in this chapter sets out to quantify how important the effect of scattering of laser light is likely to be in surgery on humans, by measuring the absorption, scattering and isotropy coefficients of fresh porcine aorta in vitro using two different techniques.

- Obtained from diffuse reflectance and transmission measurements using the standard Kubelka-Munk treatment.
- Using a scanning micro-laser reflectometer to detect the distribution of reflected light arising from scattering of a laser focussed at a point on the surface of the sample, and comparing the results with Monte Carlo simulations that take account of perpendicular irradiation and internal reflection at the boundaries.

2.2. **Materials and methods.**

Porcine aorta like human aorta consists of interwoven elastin and collagen fibres. The vessel wall is divided into three distinct layers: intima, media and adventia.

Lengths of porcine aorta were collected from the abattoir immediately after slaughter. Blood was washed from the vessels with physiological saline and the excess fat was
removed from the advential surface. To preserve the samples and prevent dehydration, the tissue was stored in physiological saline at 4°C. The wall thickness of the aorta samples varied between 1 and 1.5mm. Pig arteries were used because of their availability and similarity to human blood vessels.

2.2.1. Laterally resolved measurements.

The methodology of laterally resolved measurements using the micro scanning reflectometer have previously been documented by Oelkrug and Brun\cite{Oelkrug1977, Oelkrug1978, Oelkrug1979}. The apparatus used for these measurements is shown in figure 2.2. Coherent light emitted by a He-Ne laser $\lambda=632.8$nm (Spectra Physics) was chopped with a frequency of ~170Hz. The radiation passed through a cubic beamsplitter and was then focussed on the surface of the rotating sample by a short focal length lens. The diameter of the focussed laser spot at full width half maximum is $12.5\mu$m. To increase the degree of linear polarisation of the incident radiation, and reduce the specular reflection from the surface of the sample, two polarisers were incorporated into the set-up ($P_1, P_2$). The reflection from the surface of the sample was imaged onto the detector through the same lens and the cubic beamsplitter. The photomultiplier (Hamamatsu) with an aperture of 1mm was able to move on a linear scanner in positive and negative x directions. To improve the signal to noise ratio, the measurements were carried out with a lock-in amplifier. It is necessary to rotate the sample because the coherent radiation of the incident laser light generates a random speckle pattern\cite{SpecklePattern}, that interferes with the radial distribution of the diffusely reflected light. Rotation of the sample results in an averaging and smoothes the speckle effect. For these measurements, sections of porcine aorta were dissected axially, opened out into a flat sheet, and supported on a holder (with black background) by a metal ring. The supporting holder was connected to a simple motor enabling rotation of the sample.
Chapter Two: Determination of the optical properties of porcine vascular tissue.

Figure 2.2: Schematic of the experimental arrangement to measure the lateral light diffusion

2.2.2. Reflectance and transmission measurements.

Samples of porcine aorta were trimmed using a scalpel and mounted behind a quartz plate.

Measurements of the diffuse reflectance and diffuse transmission were carried out using a double beam spectrophotometer (Cary Model 14) fitted with an integrating sphere. Measurements were recorded over the 450-650nm wavelength range in air, with the porcine aorta placed behind the quartz plate to avoid direct contact between the moist sample and the integrating sphere.
The diffuse reflectance data was collected as follows:
Monochromatic radiation falls alternatively, perpendicularly on the sample and a Barium Sulphate standard. The light is scattered diffusely in the integrating sphere and passes through an opening onto the detector. Figure 2.3 shows the geometry used to measure the diffuse reflectance.
The 100% reflectance was measured by placing a Barium Sulphate compressed plate on the sample port, and zero reflectance $R_o$ was measured with only the holder over the sample port.

![Figure 2.3: Diagram showing geometry used to measure the diffuse reflectance $R_d$.](image)

The total transmission of the sample was measured by:
The sample and the standard were replaced with compressed Barium Sulphate discs, and the sample of porcine aorta was placed in the entrance of the reference beam, consequently all the radiation passing through reaches the walls of the integrating sphere. The standard disc in the reference beam is replaced by a 'radiation trap', and the radiation that remains unscattered by the sample is absorbed. Therefore, only the diffuse transmission of the porcine aorta is measured. Figure 2.4 shows the geometry used to measure the diffuse transmission.
Zero transmission $T_0$, was measured by placing an opaque barrier in the entrance where the sample is placed, and 100% transmission $T_{100}$ was determined by placing an empty holder in the sample beam.

![Diagram showing the geometry used to measure the diffuse transmission $T_d$.](image)

Figure 2.4: Diagram showing the geometry used to measure the diffuse transmission $T_d$.

The effects of internal reflection at the quartz/air interface were corrected for by measuring the zero reflectance $R_0$ with the quartz plate in position.

2.2.3 Analysis of data.

The scans were digitised and entered into a spreadsheet package for analysis. The results from the integrating sphere were corrected in the following way. The total corrected sample reflectance $R^c_t$ calculated using the following:

$$ R^c_t = \frac{R_s - R_0}{[R_{100} - R_0] \times [1 - 2R_Q]} \quad (2.17) $$
where

$R_s$ is the measured reflectance of the sample

$R_Q$ is the measured reflectance with the quartz plate in place.

The total transmittance $T'$, was computed using the relation:

$$T'_r = \frac{T_s - T_o}{T_Q - T_o} \quad (2.18)$$

where

$T_s$ is the measured transmission of the sample.

$T_Q$ is the measured transmission with the quartz plate in place.

Correction for the sideways diffusion of light beyond the sample port in the integrating sphere attachment of the Cary spectrophotometer was carried out as detailed by Brun.\(^{171}\)

$$D_s = \frac{1}{\pi} \frac{P}{A} \langle r \rangle \quad (2.19)$$

where

$$\langle r \rangle = \frac{\int_0^\infty rJ(r)2\pi rdr}{\int_0^\infty J(r)2\pi rdr} \quad (2.20)$$

$$D_s = \frac{1}{\pi} \frac{2L_x + 2L_y}{L_x \cdot L_y} \langle r \rangle \quad (2.21)$$

$$D_s^R = \frac{\Delta R_{corr}}{R} \quad (2.22) \quad D_s^T = \frac{\Delta T_{corr}}{T} \quad (2.23)$$

$$R_{corr} = R + \Delta R_{corr} \quad (2.24) \quad T_{corr} = T + \Delta T_{corr} \quad (2.25)$$

where

$R_{corr}$ and $T_{corr}$ are the corrected values of reflectance and transmittance taking account sideways losses.

$D_s$ is the lateral diffusion, $T$ and $R$ superscripts for the lateral diffusion for transmission and reflection respectively.

$P$ is the perimeter of the port within the integrating sphere (mm).

$A$ is the area of the port within the integrating sphere (mm\(^2\)).
$\langle r \rangle$ is the mean diffusion length of light in the sample (mm).

2.3. **Results.**

2.3.1. **Reflectance and transmission measurements.**

Measurements of the diffuse reflectance $R_d$ and diffuse transmission $T_d$ were corrected for reflection from the surface of the quartz plate placed in front of the tissue samples, and for sideways losses of light beyond the sample holder due to high sideways photon diffusion. The resulting corrected spectra were used to calculate values of the Kubelka-Munk scattering and absorption coefficients (see equations in section 2.1) of fresh porcine aorta over a wavelength range of 450 to 650 nm. Figure 2.5 shows plots of the Kubelka-Munk absorption and scattering coefficient respectively plotted as a function of wavelength. The values of the Kubelka-Munk absorption coefficient ranged from $1.6 \text{cm}^{-1}$ at 650 nm to $8.4 \text{cm}^{-1}$ at 450 nm. The Kubelka-Munk scattering coefficient shows an almost linear correlation with wavelength, increasing slightly towards the blue end of the spectrum, with values of $13.5 \text{cm}^{-1}$ at 650 nm and $17.4 \text{cm}^{-1}$ at 450 nm. An important point to note with the determination of these optical properties is that the irradiation of the sample by the light source of the Cary spectrophotometer is not diffuse. As a result the value for the diffuse transmission through the sample $T_d$ is higher than that if diffuse light were being used. This leads to a lower than expected value for the scattering coefficient of the sample. Consequently the Kubelka Munk scattering coefficient will actually be higher than $17.4 \text{cm}^{-1}$ at 450 nm.
Chapter Two: Determination of the optical properties of porcine vascular tissue.

Figure 2.5: Kubelka Munk absorption and scattering coefficients calculated from diffuse transmission and reflectance data.
Table 2.1 lists values at particular wavelengths of interest.

<table>
<thead>
<tr>
<th>Wavelength /nm</th>
<th>KM Absorption coefficient /cm⁻¹</th>
<th>KM Scattering coefficient /cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>633</td>
<td>1.7</td>
<td>13.8</td>
</tr>
<tr>
<td>514</td>
<td>3.6</td>
<td>16.1</td>
</tr>
<tr>
<td>488</td>
<td>4.8</td>
<td>16.9</td>
</tr>
</tbody>
</table>

Table 2.1. The Kubelka-Munk optical coefficients at particular wavelengths of interest

2.3.2. Laterally resolved measurements.

Laterally resolved reflection measurements were performed on four different samples of porcine aorta. Figure 2.6 shows the results of the radial distribution curves for each sample.
Chapter Two: Determination of the optical properties of porcine vascular tissue.

Figure 2.6: Normalised plots of the experimental radial intensity function $2\pi r J(r)$ for four different samples of porcine aorta.
Figure 2.7: Normalised plot of the experimental and simulation of the radial intensity function $2\pi r J(r)$ for titanium dioxide.
It is noticeable that the light diffuses over a wide range sideways from the point of irradiation (>1500μm) compared to that of titanium oxide, see figure 2.7.

The diagram shown overleaf illustrates two different radial reflectance curves for a powdered sample of titanium dioxide (Merck.Art.808). One curve is an actual experimental trace, while the other is a Monte Carlo simulation, using values for the optical properties of titanium dioxide obtained as a result of standard reflectance and transmission measurements [77]. The values of the scattering, absorption and anisotropy parameters used for this particular simulation are, 4550cm⁻¹, 0.9cm⁻¹, and 0 respectively. The principle objective of comparing these two traces is to highlight the excellent agreement between the laterally resolved reflection measurements and the simulation generated after reflection and transmission measurements. Hopefully this result emphasises the viability of using the micro-laser reflectometer for measuring the optical properties of biological materials. When comparisons were made between experimental laterally resolved reflectance curves and various Monte Carlo simulations, assuming mismatched boundary conditions (with a refractive index of 1.5), values of, $S=35\text{cm}^{-1}$, $A=1.1\text{cm}^{-1}$, and $g=0.915$ gave the best agreement. Figure 2.8 shows one experimental result compared with the simulation using these values. It is important to note that the Monte Carlo simulation was fitted to the experimental traces of the lateral light diffusion using an anisotropy coefficient of 0.915. This value was selected since it is widely accepted as a suitable representation of the anisotropy coefficient for biological tissue. Since the anisotropy and scattering parameters are related by the following equation, $S_{\text{eff}}=S(1-g)$, the value of the scattering coefficient was automatically chosen to give the best fit, with a $g$ value of 0.915. It is reasonable to assume that any combination of $S$ and $g$ would be suitable to generate a simulated curve that matched that of the experimental trace. Even assuming a totally unrealistic scenario of an anisotropy factor of zero. The only way in which a true value of the anisotropy parameter, and consequently the scattering coefficient can be determined, is by measuring the diffuse transmission of light through the sample.
Figure 2.8: Normalised plots showing the comparison between an experimental radial intensity function and a Monte Carlo simulated curve (S=35cm⁻¹, A=1.1cm⁻¹ and g=0.915).
2.3.3. Results of Monte Carlo simulations.

A series of Monte Carlo simulations were performed taking into account the two possible boundary conditions, different anisotropy factors, and variations in the absorption and effective scattering coefficients. Figure 2.9 shows two simulations obtained with identical scattering, absorption and anisotropy parameters, but comparing the differences between matched and mismatched boundaries. The difference between the refractive index of the sample and boundary, affects the shape of the relative radiance curve, as a result of total internal reflection. Rather than photons being transmitted through the tissue slab, they are reflected back into the tissue and undergo further scattering or absorption processes. Consequently photons diffuse further from the initial point of irradiation. This results in a broader relative radiance curve.
Figure 2.9: Normalised plots of Monte Carlo simulations obtained using identical optical properties, but assuming different boundary conditions.
Chapter Two: Determination of the optical properties of porcine vascular tissue.

2.4. Discussion

2.4.1. Reflectance and transmission results.
The exact description for the transfer of laser energy in tissue has been based upon the radiative transport equation \[^{[86]}\]. Because of the difficulty in solving the transport equation exactly, several approximations have been made regarding the representation of the radiance and/or the phase function. The most simple model is Beer's law, which describes the exponential attenuation of unscattered light. However, the majority of biological tissues are highly scattering, and Beer's law is not applicable. The next stage of complexity involves considering light propagating in tissue as a series of diffuse light fluxes. The model discussed by Kubelka \[^{[20,68]}\] is one such theory. This theory describes the propagation of a uniform diffuse irradiance through a one dimensional slab with no reflection at the boundaries. Two fluxes normal to the tissue surface are used, a forward flux is attenuated due to absorption and scattering, and is increased by scattering from the backward flux. The same points affect the intensity of the backward flux. The simplicity of the Kubelka-Munk model has made it a popular method for measuring the optical properties of tissue. However, assumptions of isotropic scattering, matched boundary conditions and diffuse irradiance have to be made. More complicated models including four or more diffuse fluxes have been used, even a seven flux model has been proposed. The seven flux model described by Yoon \[^{[87]}\] provides for forward and backward diffuse fluxes distributed in the x, y and z directions, in addition to a collimated light flux in the direction of the beam incidence.

In this study, measurements of the diffuse transmission \(R_d\) and diffuse transmission \(T_d\) have been used with the Kubelka-Munk formula to calculate the absorption \(A_{KM}\) and scattering coefficients \(S_{KM}\). The results of these measurements are shown in figure 2.5. It is noticeable that the absorption spectrum has peaks at 540nm and 577nm. These peaks coincide with the absorption of oxyhaemoglobin see figure 2.10. The Kubelka-Munk scattering coefficient shown in figure 2.5 shows an almost linear correlation with wavelength. However, the values are greater at shorter wavelengths.
Measurements of the optical properties of vascular material have been limited. However, three groups have measured the optical coefficients of human aorta. Van Gemert et al. found a Kubelka-Munk scattering coefficient of 6.3 cm\(^{-1}\) at 632.8 nm by conducting diffuse reflectance and transmission measurements on slabs of human aorta. In a similar study, Oraevsky et al. obtained Kubelka-Munk scattering coefficients of 16 cm\(^{-1}\) at 632.8 nm. In a slightly different study, Yoon performed diffuse transmission and goniophotometry on thin slabs of fresh human aorta. An effective scattering coefficient \(S(1-g)\) of 41.0 cm\(^{-1}\), \((S_{KM}=31\text{cm}^{-1})\) was obtained at
632.8nm. The results of the experiments performed in this chapter gave a Kubelka-Munk scattering coefficient of $17\text{cm}^{-1}$ at 632.8nm. This value compares with the value obtained by Oraevsky [89], but is somewhat higher than that reported by van Gemert [88] for human aorta, and lower than the value obtained by Yoon [87].

Direct comparison of in vitro results is difficult, and there are a number of drawbacks.

- Sample preparation and storage. The samples of aorta used by Yoon [87] were stripped to different thicknesses leaving only the intima and media layers. Additionally, the samples were stored for long durations in saline solution, removing the remaining blood from the tissue sample. Resulting in a reduction of the measured absorption coefficient.

- The treatment of internal reflection at sample boundaries certainly affects the value of the scattering coefficients. Yoon [87] fitted the asymptotic region of a plot of diffuse transmission against sample thickness to an equation that was independent of the tissue refractive index. This removed the need for any boundary conditions.

The main problem encountered during the course of the diffuse reflectance and transmission measurements was that the experimental set-up was unable to measure all the reflected and transmitted laser radiation. This was a result of high internal Fresnel reflection at the boundaries of the aorta, resulting in light travelling long distances laterally inside the sample. This radiation was undetectable at the entrance of the sample port of the reflectance attachment. As a consequence, the measured reflectance was too low, resulting in an apparent absorption coefficient that is too high, particularly in the red region of the spectrum at the long wavelength side of the haemoglobin absorption.

One drawback of these experiments was that without the measurement of collimated transmission, only the Kubelka-Munk absorption and effective scattering coefficient could be determined. These measurements failed to determine the anisotropy parameter, and were unable to show that porcine aorta causes laser radiation to be scattered in a highly forward direction. Knowledge of the anisotropy parameter is very important to determine the distribution of laser light in biological materials.
2.4.2. Laterally resolved measurements.

In these experiments the scanning micro-laser reflectometer system was used to measure the radial reflected intensity of samples of porcine aorta as a function of radial distance $r$ from the point of irradiation. The results of these measurements gave an indication to whether secondary irradiation, as a result of scattering may be an important factor for temperature rises in laser irradiated tissue. The results suggest that the light intensity millimetres away from the point of irradiation is high, and that it has a significant contribution for the temperature generation within tissue. Figure 2.6 shows the experimental plots obtained from four different samples of aorta. When compared to the lateral resolved reflectivity plots of titanium oxide, figure 2.7, it is evident that the diffusion of light sideways from the point of irradiation is significantly greater for porcine aorta. This difference is in accordance with the lower scattering coefficient of porcine aorta ($\Sigma=40\text{cm}^{-1}$). Titanium oxide has a significantly higher scattering coefficient than typical biological materials ($\text{Seff}=4550\text{cm}^{-1}$ [77] compared to $<100\text{cm}^{-1}$). In these instances, samples with high effective scattering coefficients result in photons with shorter mean paths compared with low scattering coefficients. Consequently, relative radiance curves of titanium oxide are steeper.

When comparisons were made between experimentally obtained lateral resolved reflectance curves and the plots obtained using a Monte Carlo calculation of radiative transport assuming mismatched boundary conditions, the values of the optical properties giving the best agreement were $\Sigma=35\text{cm}^{-1}$, $\text{A}=1.1\text{cm}^{-1}$ and $g=0.915$. Figure 2.8 shows the result of this Monte Carlo simulation compared with the experimentally obtained curves. A value of 0.915 was used as the anisotropy parameter as it is recognised that tissue scatters light in a highly forward direction. As a consequence of a series of fits between simulations with actual experimental traces, a scattering coefficient was selected. The value of which was determined partially by the anisotropy factor.

Although the scanning micro-laser reflectometer system has been used for the determination of the optical properties of homogeneous materials with high scattering coefficients, the determination of tissue optical properties using spatially resolved
reflectance measurements have been limited. Farrel et al [90] developed a further diffusion theory model for analysis of the radially dependent diffuse reflectance, using the shape of the reflectance curve to determine the tissue optical properties. The experimental arrangement consisted of a probe of ten 400μm diameter optical fibres placed in contact with the sample. One fibre optic acted as a source while the other fibres were used to detect the reflected light at distances of up to 10mm from the point of incident irradiation. The optical properties of various tissue phantoms, human skin and rats thigh muscle were measured in vivo. The liquid phantom consisted of solutions of various concentrations of a liquid emulsion. The optical properties derived for these solutions were within 5-10% of those determined by established techniques. The measurements on the skin and thigh muscle were reported to be consistent with the results of previous investigators.

Similarly, Groenhuis et al [91] used a reflectometer system based around a halogen lamp and optical fibre system to determine the scattering and absorption characteristics of turbid materials. Although the materials used were not identified, the method was developed to perform measurements on dental enamel. This paper [91] reported no measurements on any biological materials.

The values obtained for the optical properties of porcine aorta using the laser reflectometer system are in reasonable agreement with those of previous investigators using conventional techniques to determine the properties of human aorta. A number of measurements [87,92] have been performed at wavelengths around 630nm because the He-Ne laser operates in this region (λ=632.8nm), and because the He-Ne laser is commonly used in photodynamic therapy. Two different studies have been performed on human aorta. Yoon [87] determined absorption, scattering and anisotropy coefficients by measuring the diffuse transmission and performing goniophotometry on fresh samples of aorta. Values of A=0.52cm⁻¹ and S=41.0cm⁻¹ were reported. These results are in reasonable agreement with those obtained in this study. In a study by Keijzer et al [92], samples of aorta were frozen to prepare microtome sections. The aorta was separated into constituent layers, intima, media and adventitia, and values of the optical properties were obtained from measurements.
of diffuse transmission, reflectance and collimated transmission. Keijzer reported the following values for absorption and scattering coefficients, see table 2.2.

<table>
<thead>
<tr>
<th>Section of the aorta</th>
<th>Absorption coefficient/cm⁻¹</th>
<th>Scattering coefficient/cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intimate</td>
<td>3.6</td>
<td>171</td>
</tr>
<tr>
<td>Media</td>
<td>2.3</td>
<td>310</td>
</tr>
<tr>
<td>Adventia</td>
<td>5.8</td>
<td>195</td>
</tr>
</tbody>
</table>

Table 2.2. Results of the studies on the optical properties of human aorta performed by Keijzer [92].

The agreement between the results of Keijzer and those reported in this section particularly those of the scattering coefficient are not as good as those of Yoon [87]. One possible explanation to the differences is the way in which the aorta was stored, and separated into different layers.

The value for the anisotropy parameter obtained as a result of experiments of this chapter was uniformly large and positive. At 633nm, the anisotropy coefficient was equal to 0.91. This value was chosen along with a scattering coefficient, in order to obtain the best fit between simulation and experiment. It is similar to the values determined by previous investigators, Yoon (0.87) [87] and Keijzer (0.85, 0.9, 0.81 for intima, media and adventitia respectively) [92]. The results of this investigation confirmed that light is scattered in a highly forward direction in vascular material.

Table 2.3 summarises the results of the optical property determinations using the two different techniques described in this chapter. In addition, values of scattering, absorption and anisotropy coefficients obtained by other investigators are reported.
Diffuse reflectance and 633nm

Spatially resolved reflectance 633nm

Diffuse transmission and 633nm

goniophotometry

Table 2.3. Results of the optical properties of porcine aorta measured in this study, compared with the measurements of Yoon [87].

The results of the scattering coefficients measured using spatially resolved reflectance measurements were more in-line with those previously reported (35cm⁻¹ compared with 41cm⁻¹[87]). Values of the scattering coefficient obtained using integrating sphere methods were somewhat lower than expected because of problems experienced in detecting all transmitted and reflected radiation. The inability to detect laser radiation interacting with the tissue results in a lower than expected value for the scattering coefficient.

Figure 2.11 shows the difference in radial reflectance curves for Monte Carlo simulations obtained using the results of the two different studies. The solid line corresponds to the results obtained from the laterally resolved reflectance experiments, while the dotted line represents the results obtained from the diffuse transmission and reflectance measurements, but assumes that the porcine aorta is not an isotropic scatterer (contrary to the Kubelka-Munk conclusions). Because of differences in the scattering coefficient and the anisotropy parameter, the shapes of the radial reflectance curves were different. The trace generated using values obtained from the laser reflectometer was narrower as a result of the high effective scattering coefficient, S=35cm⁻¹ compared to 20cm⁻¹, which resulted in a lower effective path between scatterers, resulting in photons diffusing over a narrow area.
Figure 2.11: Normalised plots showing the difference between Monte Carlo simulations generated by applying the results of reflectance and transmission and radial reflectance measurements.
Chapter Two: Determination of the optical properties of porcine vascular tissue.

In conclusion to this study, two different methods have been presented for the determination of the optical properties of porcine aorta. Kubelka-Munk coefficients of absorption and scattering (effective scattering), were deduced from measurements of the integrated reflectance and transmittance using the 2-flux approximation of the diffusion theory. The simplicity of the Kubelka-Munk model has made it a popular method for measuring the optical properties of tissue. However, the technique assumes isotropic scattering, matched boundary conditions and diffuse irradiation. These conditions are not applicable when considering the interaction of laser light with biological tissue. Consequently, a novel method typically used to measure the optical properties of materials with high scattering coefficients, has been applied for measurements of biological materials, (heterogeneous substrates) with significantly lower scattering coefficients. The results of this new technique have been encouraging, with values of the reduced scattering and absorption coefficients in accordance with previous investigations \[^{[87]}\]. To enable calculation of the actual anisotropy parameter, and therefore the scattering coefficient, it is necessary to measure the diffuse transmission of light through the porcine aorta. Although measurements were confined to porcine aorta, the laser reflectometer could easily be applied to other biological materials. The results of the resolved reflectance experiments should assist investigators working to develop and optimise biomedical applications of laser/tissue interactions, by highlighting the extent to which laser radiation is distributed in irradiated tissue.
3. ASSESSMENT OF CHROMOPHORES FOR USE IN ARGON LASER ASSISTED VASCULAR ANASTOMOSIS.

3.1. Introduction.

Laser assisted vascular anastomosis relies upon laser energy to thermally coagulate blood vessel proteins in their walls to produce a 'weld' \cite{47}, sealing the vessel together, preferably without causing excessive thermal damage to the vessel outside the anastomotic region. As a result, the exact aiming of the laser beam around the anastomosis is essential. However, laser radiation is not only absorbed by the tissue, it is scattered and reflected. Consequently, rather than the laser beam behaving as a collimated beam, the laser radiation diffuses beyond the point of irradiation and the resulting heat source is spread over a wide region. The extent to which laser radiation diffuses in biological materials was the subject of the previous chapter. The scattering, absorption and anisotropy coefficients of porcine aorta were measured using a scanning micro-laser reflectometer. The results of this study confirmed that laser radiation does diffuse over a wide range from the laser spot (>1500\textmu m) and that light is strongly scattered in a forward direction. Figure 3.1 illustrates the extent of light diffusion in tissue compared to that in Titanium dioxide.
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

Figure 3.1: Comparison of the lateral light diffusion in porcine tissue and titanium dioxide.

---Porcine aorta
---Titanium dioxide

---Porcine aorta
---Titanium dioxide

Figure 3.1: Comparison of the lateral light diffusion in porcine tissue and titanium dioxide.
As a result of the sideways diffusion of laser radiation, confinement of the thermal effects around the anastomosis site is extremely difficult. One possibility is to use chromophores that selectively absorb the laser radiation, and localise the heat to a small area.

The advantage of using a chromophore to enhance the absorption of laser energy is that:

• because a greater proportion of laser radiation is being absorbed by the chromophore, the incident power required for welding can be reduced.
• the thermal effects are localised and reduce the chance of thermal damage to areas beyond the anastomosis, reducing the need for precise beam aiming.

The preferential absorption of laser radiation is demonstrated in a number of surgical procedures. In the treatment of port wine stains\(^931\), the target for absorption of the laser radiation is the haemoglobin in the blood vessels of the birth mark. Haemoglobin absorbs in the visible region of the electromagnetic spectrum, \(\lambda_{\text{MAX}} = 478, 555\text{nm}\). As a result, the Argon ion laser is selected for treatment of such complaints, because the emission wavelengths of this laser coincide with the absorption of haemoglobin. However, the Argon laser radiation is minimally absorbed by the surrounding tissue, limiting the degree of thermal damage.

A large number of dyes exist with significant absorbances at the wavelengths of the Argon ion laser. However, investigations into chromophore aided vascular anastomosis are relatively new areas of research\(^{53,59,60}\). A number of studies have been reported using externally applied chromophores to enhance the absorption of laser radiation. In the majority of cases the chromophores were selected because of their use as histological stains, (specifically dyes that attach onto connective tissue such as collagen), rather than considering their temperature generating ability, or their potential use as end-point indicators. Using a surgical stain, Basic fuchsin, (see figure 3.2 for chemical structure), Vance et al\(^{53,59}\) performed two different studies exploring the possibility of using an Argon ion laser for chromophore assisted laser anastomosis. In the first study\(^{59}\), end-to-end anastomosis of pigs coronary arteries were prepared. Application of alcoholic solutions of Basic fuchsin to the surface of
the blood vessel around the anastomosis allowed a reduction in the incident laser power, 1 watt without chromophore to 0.3-0.5 watts with chromophore. In a later study [53], longitudinal arteriotomies in the rat abdominal aorta were repaired. Basic fuchsin in alcohol was applied onto the cut vessel edges using a Rotring™ pen. The application of chromophore reduced the power density required to achieve bonding, and reduced the difficulties associated with beam aiming. Chuck et al [60] repaired abdominal aorotomies in rabbits in vivo using an Argon ion laser with or without the addition of Fluorescein isothiocyanate. The dye is orange in appearance, with an absorption maximum of 490nm. Chuck reported that the occurrence of visual changes to the blood vessel (the recognised end-point for irradiation) occurred after 15 seconds at 0.1 watts for chromophore stained aorta, compared to 15 seconds at 0.3 watts for unstained tissue. The lower energy required for vascular welding was reported to minimise the thermal damage to the surrounding healthy tissue. More recently, Poppas [64] used a human protein solder consisting of Fluorescein. A KTP laser, with an emission wavelength of 532nm was used for the in vitro repair of canine ureters. Fluorescein has a peak absorption wavelength of 495nm. Four different irradiating conditions were assessed. In group one, the ureteral edges were repaired using the laser only. In group two, the KTP laser was used in combination with 40% human albumin solder. Group three, repairs were completed using a 40% albumin solution with Iron oxide. In group four, urethers were repaired using 40% albumin with the addition of 10% Fluorescein. For group five, a 10% Fluorescein solution was applied directly on-to the surface of the vessel followed by addition of the same Fluorescein glue used in group four. The lowest energy requirement was achieved when 10% Fluorescein was used to stain the tissue before the Fluorescein glue was applied. This set of anastomoses had the highest bursting strength.

The aim of the experiments described in this section was to identify suitably absorbing chromophores for use with an Argon ion laser with application to vascular surgery. A number of chromophores were selected and were assessed using three different experimental procedures.
Spectrophotometric studies, to determine the absorption characteristics of the chromophore.  
The chromophores' temperature generating ability.  
Fluorescence studies, to measure the fluorescence quantum yields of chromophores.

3.2. **Spectrophotometric studies.**  
The principle of absorption, Beer-Lambert's law, and the measurement of the ground state absorption spectra, have been previously described in the introduction to this thesis. The aims of this study were to determine the absorption profiles of a number of different chromophores, and to obtain values for the molar decadic absorption coefficient at the Argon ion laser emission wavelengths.

3.2.1. **Materials and methods.**  
A total of ten dyes (Acidic fuchsin, Acridine orange, Basic fuchsin, Carmine, Crystal violet, Eosin Y, Erythrosin, Neutral red, Phloxin and Ponceau) with significant absorbances in the region of 500nm were selected. Each dyes' concentration was adjusted to ensure that the absorbance of each solution was within the region of detection by the spectrophotometer, (an absorbance ranging from 0 to 3). The ground state absorption spectra of each aqueous solution was recorded between 350 and 700nm using a UV-VIS spectrophotometer. The molar decadic absorption coefficient of each chromophore at the wavelengths of interest was determined by the standard procedure. A series of aqueous solutions of known concentration (c mol/dm³) were prepared from the stock chromophore powder. The absorption spectra were recorded, and the absorbances A at each wavelength (488/514nm) were noted for calculation of the absorption coefficient ε. \( A = ε c l \).

3.2.2. **Results.**  
The results of the spectrophotometry scans and the structures of each chromophore are summarised in the proceeding pages, figures 3.2-3.11. The absorption coefficients of the aqueous solutions of the chromophores are shown in table 3.1.
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Absorption coefficient 488nm cm(^{-1}) dm(^{3}) mol(^{-1})</th>
<th>Absorption coefficient 514nm cm(^{-1}) dm(^{3}) mol(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic fuchsin</td>
<td>1.7×10(^4)</td>
<td>2.3×10(^4)</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>5.2×10(^4)</td>
<td>1.7×10(^4)</td>
</tr>
<tr>
<td>Basic fuchsin</td>
<td>2.4×10(^4)</td>
<td>2.9×10(^4)</td>
</tr>
<tr>
<td>Carmine</td>
<td>5.7×10(^4)</td>
<td>7.1×10(^4)</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>1.1×10(^4)</td>
<td>2.5×10(^4)</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>2.7×10(^4)</td>
<td>7.1×10(^4)</td>
</tr>
<tr>
<td>Erythrosin</td>
<td>2.2×10(^4)</td>
<td>5.2×10(^4)</td>
</tr>
<tr>
<td>Neutral red</td>
<td>1.2×10(^4)</td>
<td>1.7×10(^4)</td>
</tr>
<tr>
<td>Phloxin</td>
<td>1.5×10(^4)</td>
<td>2.8×10(^4)</td>
</tr>
<tr>
<td>Ponceau</td>
<td>2.4×10(^4)</td>
<td>2.5×10(^4)</td>
</tr>
</tbody>
</table>

Table 3.1. Decadic absorption coefficients at the two principle Argon laser emission wavelengths, of the ten dyes studied.
Figure 3.2: Chemical structure and absorption spectrum of Basic fuchsin in aqueous solution. Concentration $3.5 \times 10^5$ mol/dm$^3$. 
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

Figure 3.3: The chemical structure and absorption spectrum of Acidic fuchsin in aqueous solution. Concentration $4.5 \times 10^3 \text{ mol/dm}^3$. 

\begin{center}
\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.3.png}
\caption{The chemical structure and absorption spectrum of Acidic fuchsin in aqueous solution. Concentration $4.5 \times 10^3 \text{ mol/dm}^3$.}
\end{figure}
\end{center}
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

Figure 3.4: Chemical structure and absorption spectrum of Acridine orange in aqueous solution. Concentration $1.6 \times 10^5$ mol/dm$^3$. 
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

Figure 3.5: Chemical structure and absorption spectrum of Carmine in aqueous solution. Concentration $1.7 \times 10^5 \text{ mol/dm}^3$. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{carmine_structure吸收光谱.png}
\caption{Chemical structure and absorption spectrum of Carmine in aqueous solution. Concentration $1.7 \times 10^5 \text{ mol/dm}^3$.}
\end{figure}
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

Figure 3.6: Chemical structure and absorption spectrum of Crystal violet in aqueous solution. Concentration $2.4 \times 10^5$ mol/dm$^3$. 

88
Figure 3.7: Chemical structure and absorption spectrum of Eosin Y in aqueous solution. Concentration $2.2 \times 10^5$ mol/dm$^3$. 
Figure 3.8: Chemical structure and absorption spectrum of Erythrosin in aqueous solution. Concentration $4.1 \times 10^5$ mol/dm$^3$. 
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

1.5
1.0
0.5
0.0
L

400
500
600
700
Wavelength/ nm

Figure 3.9: Chemical structure and absorption spectrum of Neutral red in aqueous solution. Concentration $7.8 \times 10^5$ mol/dm$^3$
Figure 3.10: Chemical structure and absorption spectrum of Phloxin in aqueous solution. Concentration $1.7 \times 10^5$ mol/dm$^3$
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

Figure 3.11: Chemical structure and absorption spectrum of Ponceau in aqueous solution. Concentration $4.8 \times 10^5$ mol/dm$^3$. 
3.3. **Temperature generation experiments.**
The aim of these experiments was to assess the temperature generating ability of a number of chromophores, by irradiating different concentrations of aqueous solutions of dyes at a fixed laser power, but varying the irradiation duration.

3.3.1. **Materials and methods.**
A series of six dyes with significant absorbances at the Argon ion laser emission wavelengths (488/514nm) were chosen. Aqueous solutions of each chromophore were prepared. The concentrations were adjusted to ensure comparable absorbances at the laser wavelengths.

The experimental arrangement used for these experiments is illustrated in figure 3.12. An Argon ion laser (Spectra Physics Model 2010, Hemel Hempstead, UK, 488/514nm) was used to irradiate a 1cm$^2$ quartz cuvette containing the solutions of different dyes. The temperature measurements immediately after irradiation were performed using an infra-red thermal imaging system (NEC San-ei Thermo tracer Model 6T-60). Infrared radiation is detected by a Cd-Hg-Te detector, liquid nitrogen was used to cool down the detector so that the thermal noise was not significant. The cuvette was scanned vertically and horizontally and a computerised thermal colour image was displayed on a video screen. Additionally, a computer program was written at Loughborough University to enable the image to be saved to disk for later analysis. The range of the instrument is -50 to 2000°C, the maximum spatial resolution 0.4mm, and the maximum temperature resolution 0.1°C.

The unfocussed beam of the Argon laser, beam size 3.0mm, was used to irradiate a 1cm$^3$ volume of dye with a series of irradiation durations at a fixed laser power, 2 watts. During irradiation the solution was stirred, so the average temperature rise within the solution was obtained.

To minimise the different temperature effects of the Argon laser wavelengths, (488,496,502,511 and 514nm) on each dye, whose extinction coefficients are different at each of these wavelengths, a monochromator was placed in front of the exit to the laser to isolate the 488nm emission wavelength.
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

3.3.2. Results.

The initial set of experiments was used to assess the temperature generating ability of dilute solutions of chromophore. However, the temperatures realised when these solutions were irradiated was minimal, and in certain cases the error of the thermal imaging camera was greater than the average temperature rise. Taking account of the errors of these measurements, at short irradiation durations there was no significant difference between the temperature generating ability of the six chromophores. However, as a result of photodegradation, as the irradiation duration was increased to 50 seconds, two particular chromophores, Basic fuchsin and Acidic fuchsin, two triphenylmethane dyes generated temperature rises higher than the remaining four dyes. The differences in the mean temperature rise was noticeable even when the errors of the measurements were taken into consideration. Consequently, further
temperature generation experiments were performed on these chromophores. A laser power of 2 watts, and irradiating durations of 30 and 40 seconds were used. However, a wider range of dye concentrations were investigated. Table 3.2 shows the results of the temperature generation experiments on solutions of Basic and Acidic fuchsin.
<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Concentration mol/dm³</th>
<th>Absorbance 488nm</th>
<th>Irradiation duration seconds</th>
<th>MeanΔT °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuchsin</td>
<td>2.2x10^{-5}</td>
<td>0.53</td>
<td>30</td>
<td>6±1</td>
</tr>
<tr>
<td></td>
<td>2.2x10^{-5}</td>
<td>0.53</td>
<td>40</td>
<td>8±1</td>
</tr>
<tr>
<td></td>
<td>3.3x10^{-5}</td>
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<td>30</td>
<td>8±1</td>
</tr>
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<td>3.3x10^{-5}</td>
<td>0.80</td>
<td>40</td>
<td>10±1</td>
</tr>
<tr>
<td></td>
<td>4.4x10^{-5}</td>
<td>1.1</td>
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<td>8.8x10^{-5}</td>
<td>2.1</td>
<td>40</td>
<td>14±1</td>
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<td>Acidic fuchsin</td>
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<td>0.3</td>
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<td>4±1</td>
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<td>5±1</td>
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<td>2.9x10^{-5}</td>
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<td>6±1</td>
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<td>8±1</td>
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<td>10±1</td>
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<td>9±1</td>
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<td>12±1</td>
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<td>9.5x10^{-5}</td>
<td>1.6</td>
<td>40</td>
<td>14±1</td>
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</tbody>
</table>

Table 3.2 Mean temperature increases of a range of aqueous solutions of Basic and Acidic fuchsin, irradiated at a power of 2 watts for durations of 30 and 40 seconds.
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

The results of this more detailed study into the temperature generating ability of Basic and Acidic fuchsin using a range of chromophore concentrations concluded that there was no significant difference between the average temperature rises of these two different dyes. (See table 3.3).

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Absorbance at 488 nm</th>
<th>Mean ΔT °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuchsin</td>
<td>0.53</td>
<td>6±1</td>
</tr>
<tr>
<td>Acidic fuchsin</td>
<td>0.5</td>
<td>6±1</td>
</tr>
<tr>
<td>Basic fuchsin</td>
<td>0.8</td>
<td>8±1</td>
</tr>
<tr>
<td>Acidic fuchsin</td>
<td>0.8</td>
<td>8±1</td>
</tr>
<tr>
<td>Basic fuchsin</td>
<td>1.3</td>
<td>9±1</td>
</tr>
<tr>
<td>Acidic fuchsin</td>
<td>1.3</td>
<td>10±1</td>
</tr>
</tbody>
</table>

Table 3.3. Table showing similarities between the mean temperature rises of matched solutions of Basic and Acidic fuchsin, irradiated at a power of 2 watts for 30 seconds.

For the final set of experiments in this section, the temperatures resulting from more concentrated solutions were measured. A fixed laser power of 2 watts was used with an irradiation duration of 50 seconds.
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Concentration mol/dm³</th>
<th>Absorbance before</th>
<th>Absorbance after</th>
<th>Mean ΔT/°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuchsin</td>
<td>1.5x10⁻³</td>
<td>-</td>
<td>-</td>
<td>24±3</td>
</tr>
<tr>
<td>Acidic fuchsin</td>
<td>8.8x10⁻⁴</td>
<td>-</td>
<td>-</td>
<td>26±3</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>3.7x10⁻⁴</td>
<td>-</td>
<td>-</td>
<td>20±3</td>
</tr>
<tr>
<td>Erythrosin</td>
<td>4.5x10⁻⁴</td>
<td>-</td>
<td>-</td>
<td>23±3</td>
</tr>
<tr>
<td>Phloxin</td>
<td>6.7x10⁻⁴</td>
<td>-</td>
<td>-</td>
<td>20±3</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>1.9x10⁻⁴</td>
<td>-</td>
<td>-</td>
<td>21±3</td>
</tr>
<tr>
<td>Basic fuchsin</td>
<td>1.2x10⁻⁴</td>
<td>3.0</td>
<td>2.9</td>
<td>22±3</td>
</tr>
<tr>
<td>Acidic fuchsin</td>
<td>1.7x10⁻⁴</td>
<td>3.1</td>
<td>3.0</td>
<td>23±3</td>
</tr>
<tr>
<td>Eosin Y*</td>
<td>1.1x10⁻⁴</td>
<td>3.1</td>
<td>0.3</td>
<td>7±3</td>
</tr>
<tr>
<td>Erythrosin*</td>
<td>1.4x10⁻⁴</td>
<td>3.1</td>
<td>0.7</td>
<td>15±3</td>
</tr>
<tr>
<td>Phloxin*</td>
<td>2.0x10⁻⁴</td>
<td>3.0</td>
<td>0.3</td>
<td>10±3</td>
</tr>
<tr>
<td>Acridine orange*</td>
<td>5.7x10⁻⁵</td>
<td>3.0</td>
<td>0.2</td>
<td>16±3</td>
</tr>
<tr>
<td>Basic fuchsin</td>
<td>4.1x10⁻⁵</td>
<td>1.1</td>
<td>1.0</td>
<td>16±3</td>
</tr>
<tr>
<td>Acidic fuchsin</td>
<td>5.9x10⁻⁵</td>
<td>1.0</td>
<td>1.0</td>
<td>16±3</td>
</tr>
<tr>
<td>Eosin Y*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erythrosin*</td>
<td>4.9x10⁻⁵</td>
<td>1.0</td>
<td>0.24</td>
<td>7±3</td>
</tr>
<tr>
<td>Phloxin*</td>
<td>6.9x10⁻⁵</td>
<td>1.0</td>
<td>0.15</td>
<td>7±3</td>
</tr>
<tr>
<td>Acridine orange*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(* Evidence of fading of the chromophore as a result of irradiation)

Table 3.4. Mean temperature increases observed in a range of highly absorbing solutions of chromophore, irradiated at a power of 2 watts for 50 seconds.

During the course of these experiments, the ground state absorption spectra of each chromophore was recorded before and after irradiation. These measurements were performed as a result of preliminary temperature generation experiments where fading of the chromophore was observed. The footnotes of table 3.4 indicate that the temperature generating ability of Eosin Y, Erythrosin, Phloxin and Acridine orange was affected by the photofading of the chromophores. Figures 3.13-3.16 illustrate the reduction in absorbance of these four different chromophores.
Figure 3.13: The change in absorbance of Eosin Y as a result of irradiation, 2 watts, 50 seconds.
Chapter 11: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

Figure 3.14: The change in absorbance of Erythrosin as a result of irradiation, 2 watts, 50 seconds.
Figure 3.15: The change in absorbance of Phloxin as a result of irradiation, 2 watts, 50 seconds.
Figure 3.16: The change in absorbance of Acridine Orange as a result of irradiation, 2 watts, 50 seconds.
3.3.3. **Discussion.**

The primary objective of this particular investigation was to confirm that the mode of action of the chromophores was principally thermal, and that the dyes underwent limited photochemical reactions. Studies into the potential temperature generating ability of chromophores have been reported by Vance [95]. In order to investigate which dyes gave the highest transformation from absorbed laser energy to heat, the temperature reached within the dyes during irradiation, and the degree of fluorescence were measured. Equal amounts of alcoholic solutions of dye were dropped onto separate filter papers, allowed to spread, and were irradiated at a power of 0.5 watts for ≈1 second. The resulting temperature increases were measured by a thermocouple placed under several pieces of filter paper to avoid direct absorption of the laser by the thermocouple. The amount of fluorescence was measured [95] in a purely qualitative manner by observing the glow of each dye during irradiation. The degree of fluorescence was measured on a arbitrary scale of 1 to 3. Sixteen histological stains with significant absorption around 514nm were studied. The results of temperature and subjective fluorescence studies concluded that Basic fuchsin was the most suitable dye. It gave the highest absorption at 514nm, the highest temperature rise, along with two other dyes, Methyl violet and Bismark brown, and showed little fluorescence.

The results of the temperature generation experiments are summarised in tables 3.2-3.4. Throughout these investigations, if the large errors in the measurements were taken into account, there appeared to be limited differences in the temperature generating ability of the six chromophores studied. When dilute solutions of chromophore with optical densities of 0.25 at 488nm were irradiated under identical conditions, for short irradiation durations, there was no difference between the mean temperature rise of the six dyes. However, when the irradiation duration was increased to 50 seconds, Basic fuchsin and Acidic fuchsin gave mean temperature rises higher than those of the remaining chromophores. One likely explanation is that
Basic fuchsin and Acidic fuchsin are the only two dyes of the six studied that did not fade appreciably during irradiation. Consequently, their temperature generating abilities appeared to be significantly higher. Basic and Acidic fuchsin have very low quantum yields of fade, and they do not fluorescence significantly, therefore it is reasonable to assume that their excited state are very short lived. Because of the rather surprising result, where there was a suggestion that at higher irradiation durations the two fuchsin were the most efficient heat generators, a further study was undertaken to investigate the temperature generating ability of Basic/Acidic fuchsin, using a range of dye concentrations. As the concentration of the dyes were increased from $2 \times 10^{-5}$ mol/dm$^3$ to $9.5 \times 10^{-5}$ mol/dm$^3$, there was a corresponding increase in absorbance. The resulting temperature rise increased accordingly. The temperature generation as a function of chromophore concentration plateaued as the dye concentration increased (see figure 3.17) as there was a limit to the amount of photons absorbed by the solution, and due to convective cooling of the irradiated solutions by the surrounding air. Comparing solutions of Basic fuchsin and Acidic fuchsin with matched absorbances, (see table 3.3 in the results section), it is noticeable that there is little difference between the temperature generating ability of the two fuchsin dyes.
Figure 3.17: The temperature generating ability of Basic fuchsin as a function of dye concentration.

From the aspect of absorption of laser energy, there was reasonable agreement between the temperature rises recorded during this study, and those of the previous investigation, when lower concentrations of chromophore were explored. In the final set of temperature generation experiments, highly absorbing solutions of chromophores were assessed. The optical densities of each dye solution at 488nm were matched to 1, 3 and 10. Taking into account the errors associated with the
infra-red thermal camera, in accordance with the previous investigation, there appeared to be no significant difference of the six chromophores studied. The mean temperature rise observed for each dye are shown in table 3.5.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Mean temperature rise /°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuchsin</td>
<td>24±3</td>
</tr>
<tr>
<td>Acidic fuchsin</td>
<td>26±3</td>
</tr>
<tr>
<td>Erythrosin</td>
<td>23±3</td>
</tr>
<tr>
<td>Phloxin</td>
<td>20±3</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>21±3</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>20±3</td>
</tr>
</tbody>
</table>

Table 3.5. Mean temperature increases of highly absorbing chromophore solutions (Absorbance ≈10), irradiated at a power of 2 watts for 50 seconds.

At lower dye concentrations, comparisons of the temperature generating ability of the chromophores was difficult, because of the photochemical fading of Eosin Y, Phloxin, Erythrosin, and Acridine orange. Figures 3.13-3.16 show the change in absorbance of each chromophore as a result of irradiation. For low concentration dye solutions, a 50% change in optical density had a significant affect on the resulting temperature rise. Conversely, the effects of chromophore fading were insignificant for more concentrated dye solutions, as a 50% reduction in concentration had little effect on the percentage absorption of the laser radiation.

During the course of these experiments, various dyes demonstrated fluorescence, particularly Eosin Y, Phloxin and Acridine orange. Fluorescence is a radiative transition. After the initial absorption of radiation, the energy of an electronically excited state may be lost in a number of different ways. Commonly, energy is transferred into rotation, vibration and translation of the surrounding molecules, resulting in a temperature increase of the environment. However, it is possible that the energy of the electronically excited state can survive long enough to undergo spontaneous emission, emitting the excess energy as radiation. This electronic
transition is known as fluorescence. It would be reasonable to expect that chromophores that exhibit fluorescence would have a lower temperature generating ability. Rather than the excess electronic energy being lost in a non-radiative transition (ie heat), the energy is emitted as radiation. The possible relationship between the degree of fluorescence of a chromophore and its temperature generating ability will be the subject of discussion in section 3.4.

3.4. Fluorescence quantum yield measurements.

For photochemical processes, the measure of the efficiency of a particular route is described by a quantum yield. This quantifies the yield of a particular product relative to the amount of light energy put into a reaction mixture.

Consider a general photochemical reaction:

$$A + h\nu \rightarrow B$$

the quantum yield for the production of B can be written as

$$\Phi_B = \frac{\text{No of molecules of } B \text{ formed per unit time per unit volume}}{\text{No of quanta absorbed per unit time per unit volume}}$$

(3.1)

The definition of a quantum yield is a straightforward one. If A converts to species B each time it absorbs a photon, then the quantum yield for the formation of B is equal to 1, and the reaction has a 100% efficiency. However, if species A has a number of possible reaction pathways, which do not lead to product B, the quantum yield is reduced and is less than 1.

In addition to defining a quantum yield for a particular photochemical reaction, the efficiency of any process can be defined, including photophysical events such as fluorescence and phosphorescence. A quantum yield for fluorescence can be described by:
\[ \Phi_F = \frac{\text{No of quanta emitted per unit time per unit volume}}{\text{No of quanta absorbed per unit time per unit volume}} \] (3.2)

The number of photons per second that are absorbed or emitted as fluorescence is related to the intensity. As a result, the fluorescence quantum yield can be written as:

\[ \Phi_F = \frac{I_F}{I_{abs}} \] (3.3)

where \( I_F \) is the fluorescence intensity and \( I_{abs} \) is the intensity of the absorbed radiation. This equation is suitable when radiation is supplied continuously, ensuring a steady state concentration of the excited state, and producing a constant fluorescence intensity. Investigations into the fluorescence yields of chromophores to be used to enhance absorption of laser radiation, for application to vascular surgery have been limited. Considerations of a chromophores photophysical properties have largely been ignored when selecting suitably absorbing dyes.

3.4.1. Materials and methods.
Details on the measurements of emission spectra have been described in a previous chapter. The fluorescence emission spectra of Acidic fuchsin, Acridine orange, Basic fuchsin, Eosin Y, Erythrosin and Phloxin were measured using a Spex fluorimeter. For each chromophore, a dilute aqueous solution with an absorbance of less than 0.02 at the exciting wavelength was used. If a solution with a concentration \( c \) (mol/dm\(^3\)), with a molar absorption coefficient \( \varepsilon \) (cm\(^{-1}\)dm\(^3\) mol\(^{-1}\)), and a fluorescence quantum yield of \( \Phi_F \) is placed in a monochromatic light beam of incident intensity \( I_o \), the total fluorescence intensity is given by:

\[ I_F = I_o \left(1 - 10^{-\varepsilon c} \right) \Phi_F \] (3.4)
3.4.2. **Results.**

The results of the fluorescence quantum yield measurements are shown in table 3.6. The quantum yields of the standard compounds are also included in the table. The fluorescence spectra of each chromophore and standard compounds are shown in figures 3.18-3.23. For each chromophore, the fluorescence emission spectra were studied as a function of excitation wavelength to determine if the fluorescence quantum yield was wavelength dependant.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Standard</th>
<th>Excitation λ/nm</th>
<th>$\Phi_F$ Standard</th>
<th>$\Phi_F$ Chrom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine orange</td>
<td>Rhodamine 590</td>
<td>455</td>
<td>0.86</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>488</td>
<td>0.86</td>
<td>0.25</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>Rhodamine 590</td>
<td>470</td>
<td>0.86</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>480</td>
<td>0.86</td>
<td>0.20</td>
</tr>
<tr>
<td>Phloxin</td>
<td>Rhodamine 590</td>
<td>490</td>
<td>0.86</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>0.86</td>
<td>0.19</td>
</tr>
<tr>
<td>Erythrosin</td>
<td>Rhodamine 590</td>
<td>470</td>
<td>0.86</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td>490</td>
<td>0.86</td>
<td>0.02</td>
</tr>
<tr>
<td>Acidic fuchsin</td>
<td>Rhodamine B</td>
<td>500</td>
<td>0.61</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>480</td>
<td>0.61</td>
<td>0.006</td>
</tr>
<tr>
<td>Basic fuchsin</td>
<td>Rhodamine B</td>
<td>530</td>
<td>0.61</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

Table 3.6. Quantum yield of fluorescence of aqueous solutions of chromophore obtained by ratio with standard compounds.
Figure 3.18: The normalised fluorescence spectrum of Acridine Orange and Rhodamine 590. (Inset- The fluorescence spectra of Acridine Orange excited at 455 and 488nm).
Figure 3.19: The normalised fluorescence spectrum of Eosin Y and Rhodamine 590. (Inset- The fluorescence spectra of Eosin Y excited at 470, 480, 490 and 500nm).
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

1.2

- Rhodamine 590
- Phloxin

Figure 3.20: The normalised fluorescence spectrum of Phloxin and Rhodamine 590. (Inset- The fluorescence spectra of Phloxin excited at 500, 510, 520 and 530nm)
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

Figure 3.21: Normalised fluorescence spectrum of Erythrosin and Rhodamine 590. (Inset- The fluorescence spectra of Erythrosin excited at 480, 490, 500 and 510nm).
Figure 3.22: Normalised fluorescence spectrum of Acidic fuchsin and Rhodamine B.
(Inset - The fluorescence spectra of Acidic fuchsin excited at 470, 480, 490 and 500 nm).
Chapter Three: Assessment of chromophores for use in Argon laser assisted vascular anastomosis.

Figure 3.23: The normalised fluorescence spectrum of Basic Fuschin
3.5. **Discussion.**

In accordance with the observations made during the temperature generation experiments, when intense fluorescence was evident from a number of chromophores, Acridine orange, Eosin Y and Phloxin were the three dyes with the highest fluorescence quantum yields, (0.24, 0.21 and 0.2 respectively). A series of experiments was undertaken to determine the wavelength dependency of the quantum yield. The insets of figures 3.18-3.22 show how the fluorescence emission spectra of the dyes is independent of excitation wavelength. There is good agreement between the results obtained in the current study and those of previous investigations, see table 3.7. A value for the fluorescence quantum yield of Phloxin in aqueous solution could not be found in the literature. Consequently a further measurement was performed, and a value of the quantum yield of fluorescence was determined from a singlet lifetime measurement using single photon timing methods (see section 1.6.4), $\tau_s=1.37\text{ns}$, and from the natural radiative lifetime $\tau_0$, determined from the Strickler Berg relationship \([96]\), which is given by:

$$
\frac{1}{\tau_0} = 2.880 \times 10^{-9} n^2 \langle \tilde{\nu}^{-3} \rangle_{AV^{-1}} \int \varepsilon d \ln \tilde{\nu} \tag{3.5}
$$

where

$$
\langle \tilde{\nu}^{-3} \rangle_{AV^{-1}}
$$

is the reciprocal of the mean value of the wavenumber to the power -3 in the fluorescence spectrum,

$$
\int \varepsilon d \ln \tilde{\nu}
$$

is the integrated absorption.

$n^2$ is the square of the mean refractive index of the bulk solvent,

and $g_u$ and $g_s$ is the multiplicity of the upper and lower states,

Past research \([97,98]\) studying the effect of halogenation of fluorescein dyes have suggested that the decrease in fluorescence yield from fluorescein (0.92) \([99]\) to Eosin (0.22) \([100]\) and Erythrosin (0.02) \([101]\) is due not only to an increased intersystem
crossing rate, but also to an increased internal conversion rate. Values for the quantum yield of fluorescence of the two triphenylmethane dyes, Basic fuchsin and Acidic fuchsin could not be obtained from the literature. Studies \(^{102}\) into the fluorescence yields of a range of triphenylmethane dyes have revealed that the fluorescence exhibited by this class of dye is very weak when in solvents of low viscosity, and that the lifetimes of the fluorescing states are of the order of picoseconds. However, stronger fluorescence is observed in highly viscous media \(^{103}\).

<table>
<thead>
<tr>
<th>System</th>
<th>(\Phi_f) Chromophore (^{[ref]})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine orange in ethanol</td>
<td>0.20 (^{104})</td>
</tr>
<tr>
<td>Eosin Y in water</td>
<td>0.22 (^{100})</td>
</tr>
<tr>
<td>Phloxin in water</td>
<td>0.21*</td>
</tr>
<tr>
<td>Erythrosin in water</td>
<td>0.02 (^{101})</td>
</tr>
</tbody>
</table>

* Obtained from singlet lifetime and natural radiative lifetime measurements

Table 3.7. Quantum yields of fluorescence of Acridine orange, Eosin Y, and Erythrosin obtained from the literature.

It is noticeable from table 3.6 in the results section, that the quantum yield of fluorescence of Basic fuchsin is written as \(<0.005\) rather than an actual value. Consequently, it was difficult to calculate the quantum yield by ratio of the areas underneath the fluorescence spectra. The fluorescence emission spectra of Acidic and Basic fuchsin were obtained using a combination of apparatus settings, and solution concentrations that would be inappropriate for measuring the quantum yield of fluorescence. For this reason, the quantum yield of fluorescence of Basic fuchsin was quantified as being much less than that of Acidic fuchsin.

The temperature rises as a result of the irradiation of a chromophore are as a consequence of radiationless transitions. However, fluorescence is a radiative transition. Such transitions do not result in a temperature increase of the environment. Consequently, the heat developed as a result of irradiation is
proportional to $1-\phi_F$. As the fluorescence yield increases, the temperature generating ability of the chromophore decreases accordingly. However, because the temperature rise is proportional to $1-\phi_F$, there has to be a significant difference in the degree of fluorescence to affect the temperature generating ability of the chromophore.

The emission measurements of this set of chromophores revealed that the fluorescence quantum yields ranged from 0.005 (Acidic fuchsin) to 0.24 (Acridine orange). For this difference in fluorescence quantum yield, it would be expected that there would be a difference in the temperature generating ability of these dyes. When highly absorbing solutions were irradiated at a power of 2 watts for a duration of 50 seconds, the average temperature rises of Basic fuchsin and Acridine orange were 26 and 21°C respectively. Taking into account the degree of error associated with the temperature measurements ≈10%, there is a good correlation between the fluorescence quantum yield of the dyes, and the average temperature rises when irradiated. For those chromophores with high fluorescence yields, Acridine orange (0.25), Eosin Y (0.22) and Phloxin (0.20), the resultant temperature rises following irradiation were of the order 20% lower than those dyes with low fluorescence yields, Erythrosin (0.018), Acidic fuchsin (0.005) and Basic fuchsin (<0.005) (see tables 3.4 and 3.5). Furthermore, the values quoted for the fluorescence quantum yields would be expected to be the maxima, since the fluorescence measurements were performed using dilute solutions. More concentrated solutions, for example those used during the course of the temperature generation measurements, might well have slightly lower fluorescence yields due to concentration quenching. In conclusion, the primary objective of this study was to confirm that the chromophores selected could be used as efficient energy converters, and that they exhibited limited photochemical activity. Several dyes studied rapidly photobleached following irradiation. This high quantum yield of fade made them unsuitable for use as potential chromophores. Three different experimental procedures have been used to assess the suitability of a number of different chromophores to enhance the absorption of laser radiation for use in laser assisted vessel anastomosis. The ground state absorption profiles and molar decadic absorption coefficients at the Argon ion emission wavelengths have been determined.
A series of temperature generation experiments have been performed to determine if there was a difference between the temperature generating ability of the chromophores. Additionally, the fluorescence quantum yield of each chromophore was measured to confirm the results of the temperature generation experiments. All dyes studied were strongly absorbing at the Argon laser emission wavelengths, and realised temperature rises in the region of 20°C when irradiated at 2 watts for 50 seconds. Taking into account the error associated with the thermotracer system, the difference in fluorescence quantum yield of the dyes was borne out in the differences in the temperature generating ability of the chromophores (≈20%) (Heat ∝ (1−Φf)).
4. **BURSTING PRESSURE STUDY I. LONGITUDINAL ARTERIOTOMY REPAIR BY LASER.**

4.1. **Introduction.**

The mechanical strengths of vascular anastomosis repaired by laser have been studied by several research groups [41,51,59]. An Argon ion laser was used by Vance [59] to perform anastomoses in pig coronary arteries, mechanical bursting pressure tests were used to determine the strengths of the resulting welds.

In a similar study by Fenner [105,106], bonds between slices of sheep aorta were produced in a purely thermal manner using radiation from a 120W lamp to heat a perspex clamp supporting the vessels. The bond quality was assessed as a function of bonding temperature by determining their breaking shear strength per unit area.

The objectives of this first bursting pressure study were as follows:

- To gain experience of a new technique, which is particularly sensitive to operator error.
- To compare the strength of anastomosis repaired by the Argon ion laser with and without the addition of a suitably absorbing chromophore.
- To explore the possibility of combining various proteins to the chromophore to act as a human solder.

The use of chromophores to enhance absorption at the anastomotic site have been studied by Chuck [60] and Vance [53,59]. Additionally, several groups have reported the use of protein based glues for a range of surgical procedures [107,108,109,110]. However, research using chromophores combined with various ‘tissue glues’ is a relatively new development. In a more recent study Poppas [94] used a mixture of human albumin combined with fluorescein to repair canine ureters.

In the present study three different experimental groups were used.

- No additives.
- With bovine albumin.
- With bovine albumin and chromophore.
Longitudinal arteriotomies were repaired and the quality of the welds were assessed by bursting pressure. To establish the optimum irradiating conditions, the bursting strength was studied as a function of incident laser power, irradiating duration and, where applicable, bovine albumin concentration and chromophore.

4.2. Materials and Methods.
Porcine splenic arteries were collected from the abattoir and were used immediately after collection. Splenic arteries were used because they are straight arteries, have few side branches, and are a similar diameter to human coronary arteries. The advential surface of the artery was cleared of fat and the vessel was washed with saline solution to remove any traces of blood. The vessel lengths were supported on a stainless steel high pressure gas tube. Tiny holes were drilled in the gas tube in the area of vessel overlap to assist in the testing of bursting pressure. A longitudinal arteriotomy of length 2 cm was prepared, the cut edges were surface dried, and two horizontal mattress sutures (Surgibond™ 5/0) were inserted at either end of the slit to evert the vessel edges. The gas tube supporting the vessel was clamped in place to ensure that there was minimal movement of the artery during irradiation. The laser was used continuously, and the repair was effected as a series of ‘spot welds’ along the length of the arteriotomy, for each slit approximately 10 spot welds were fashioned. The areas were irradiated until a recognisable end-point had been reached, characterised by a glowing of the tissue[^9][^11].

A collinear optical system was constructed using an Argon ion laser (Spectra Physics Model 2010, Hemel Hempstead, UK, 488/514nm) and a Helium Neon laser (Spectra Physics Model 102-4). The later was used to ensure that a laser beam would be visible through laser safety goggles. The beams were focused into a 600 micron diameter quartz optical fibre (Newport Co.). The beam emerging from the distal end of the fibre was found to be too divergent for use. Consequently a hand piece containing a fibre optic holder and converging lens (FPH-DJ Newport Co.) was constructed enabling variation of the spot size. This system is illustrated diagrammatically in figure 4.1. A range of laser powers was used during the sealing
process in order to determine the most suitable power for successful repair. The laser powers ranged from 0.1W to 0.3W. Irradiation durations of less than 5 seconds per spot were applicable for the highest laser powers, whereas significantly longer irradiation times (~20 seconds per spot) were used with the lowest powers. In each case a spot size of 1mm was used. Table 4.1 gives details of the laser parameters used during these experiments.

![Figure 4.1: Schematic showing the optical system coupling the Argon and Helium-Neon laser beams for use in effecting laser anastomosis](image)

<table>
<thead>
<tr>
<th>Irradiating condition</th>
<th>Spot diameter</th>
<th>Power</th>
<th>Irradiation duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon only</td>
<td>1mm</td>
<td>0.1-0.3W</td>
<td>20 sec/spot</td>
</tr>
<tr>
<td>Argon + bovine albumin</td>
<td>1mm</td>
<td>0.1-0.3W</td>
<td>20 sec/spot</td>
</tr>
<tr>
<td>Argon+bovine albumin+chromophore</td>
<td>1mm</td>
<td>0.1-0.3W</td>
<td>&lt;5-15 sec/spot</td>
</tr>
</tbody>
</table>

Table 4.1. Laser parameters employed in these experiments.

Four studies were undertaken. In addition to varying the irradiation condition, the quality of arteriotomy repair was assessed as a function of the following variables.
- Incident laser power.
• Concentration of bovine albumin.
• Chromophore added to bovine albumin to create a laser-activated glue.

4.2.1. Bovine albumin.
Powdered bovine albumin (Aldrich Chemical Company Ltd, Poole, UK) was used to prepare solutions ranging in concentration from 20% w/w to 70% w/w. The powdered albumin was added slowly to a stirred solution of Ringers saline solution to ensure thorough mixing.

4.2.2. Chromophores.
Four different chromophores were used in combination with bovine albumin to create laser activated glues: Basic fuchsin, Erythrosin, Eosin Y and Phloxin (see chapter 3 for details). The concentration of dye was adjusted to ensure comparable optical densities amongst the different albumin/chromophore mixtures. Typically the dye concentration within the glue was in the region of 2-4×10^-2 mol/dm^3. The albumin and albumin/dye mixtures were applied to the cut edges of the longitudinal arteriotomy using a syringe and fine needle to produce a thin film between and around the everted slit.

4.2.3. Measurement of bursting pressure.
Immediately following repair each arteriotomy was subjected to a series of bursting pressure tests. To prevent damage to the artery during manipulation the same gas needle used as a support during irradiating was fixed onto the pressure testing device as shown in figure 4.2. Using a syringe driven by a calibrated infusion pump and a length of manometer tubing, Ringers solution was injected into the artery. The resulting pressure increase was measured by a pressure transducer (RS components, No341-979), the output from which was amplified and saved to disk. The pressure transducer was regularly calibrated using a water manometer. Ringers saline solution was injected into the artery until the pressure build-up caused the first major leak. The bursting pressure was assumed to be the highest measured pressure before the arteriotomy burst.
Chapter Four: Bursting pressure study. Longitudinal arteriotomy repair by laser.

I. Longitudinal arteriotomy repair by laser.

Calibrated infusion pump

Femoral artery

Pressure transducer

MS-DOS computer

Amplifier

Figure 4.2: Diagram showing the experimental details for measuring the burst pressure of longitudinal arteriotomies repaired by laser

4.3. Results.

The results of the three bursting pressure studies are presented in the proceeding sections.

4.3.1. Variation in the incident laser power.

4.3.1.1. Argon ion laser. No bovine albumin or chromophore.

The results of the mechanical burst tests for longitudinal arteriotomies repaired at five different power settings are shown in table 4.2.
Few of the repaired arteriotomies were able to withstand pressures comparable with typical intraluminal pressures. At no stage during lasing was there any evidence of visual change in the irradiated vessel. This suggested that the coagulation temperature had not been reached, and that the combinations of power density and irradiation duration were insufficient to cause tissue fusion. The maximum burst pressure obtained at any power density in this group of arteriotomies was 80mmHg. A rather striking observation was that at a power of 0.2 watts the arteriotomy was only able to withstand a pressure of 2mmHg. This is not a true reflection of the strength of vessels repaired at this laser power, it is more likely a consequence of operator error.

4.3.1.2. Argon ion laser with bovine albumin. No chromophore.

The results from this group of arteriotomy repairs are shown in table 4.3. The bovine albumin solution was prepared with a concentration of 40% w/w.

<table>
<thead>
<tr>
<th>P (watts)</th>
<th>Power density (watt/cm²)</th>
<th>Irradiation duration (sec/spot)</th>
<th>Mean bursting pressure (mmHg)</th>
<th>Range of bursting pressures (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>19</td>
<td>20</td>
<td>32.2</td>
<td>25-37</td>
</tr>
<tr>
<td>0.2</td>
<td>26</td>
<td>20</td>
<td>23.8</td>
<td>21-28</td>
</tr>
<tr>
<td>0.25</td>
<td>32</td>
<td>20</td>
<td>25.7</td>
<td>24-29</td>
</tr>
<tr>
<td>0.3</td>
<td>38</td>
<td>20</td>
<td>25.7</td>
<td>15-38</td>
</tr>
</tbody>
</table>

Table 4.3. Table showing the burst pressures obtained for the arteriotomies repaired with bovine albumin.
Chapter Four: Bursting pressure study I. Longitudinal arteriotomy repair by laser.

The results of the bursting pressure tests on this set of welds was poor because the temperatures required for blood vessel protein coagulation, leading to tissue fusion were not achieved, because in the absence of dye, the tissue and bovine albumin were unable to absorb sufficient laser radiation. The range of bursting pressures for the arteriotomies repaired with Argon ion laser and bovine albumin was 15-38mmHg, compared with a range of 0-80mmHg for the arteries repaired using the Argon laser on its own. The similarities in mean bursting pressure in these two sets of experiments can be explained by the fact that the addition of bovine albumin to the surface of the artery makes little difference to the amount of laser radiation absorbed. Aqueous solutions of bovine albumin prepared for use in this group of experiments were almost colourless except for a slight yellow shade. Inspection of the absorption spectrum of bovine albumin reveals that these solutions have minimal absorption in the visible region of the electromagnetic spectrum. (see figure 4.3). At each power density, although surface drying of the vessel edges and albumin was apparent, there was no sign of coagulation of the tissue. This suggested that the temperature required for blood vessel fusion had not been achieved.
Chapter Four: Bursting pressure study of Longitudinal arteriotomy repair by laser.

2.0
1.5
1.0
0.5
0.0

Absorbance

300 400 500
Wavelength/ nm

Figure 4.3: The absorption spectrum of bovine serum albumin.

4.3.1.3. Argon ion laser with bovine albumin and chromophore.

The results from this group of vessel repairs are shown in table 4.4. The laser activated glue consisted of bovine albumin 40% w/w and the chromophore Basic fuchsin.
Table 4.4. Table showing the burst pressure obtained for the arteriotomies repaired with bovine albumin and Basic fuchsin.

In this experiment the strength of the repairs assessed by bursting pressure were beginning to approach values comparable with suprasystolic pressures. At each power density lasing was continued until a visible change in the tissue surface was observed. This came in the form of an intense glowing of the tissue corresponding to an increase in the back-scattered radiation from the surface of the vessel. This corresponds to coagulation of tissue proteins\textsuperscript{[39,111]} and is the best indication of an end point for irradiation. The irradiation durations for this particular set of welds ranged from less than 5 seconds per spot to 20 seconds per spot. When low laser powers were used, the onset of tissue coagulation was easily controllable and developed over a number of seconds. Conversely, where higher powers were employed, it was more difficult to control the extent of tissue coagulation, which developed almost instantaneously. Irradiation beyond this stage caused surface carbonisation of the tissue, resulting in a weaker bond, (0.3W, < 5sec/spot , mean bursting pressure=42.6mmHg). The optimum power density employed in this set of arteriotomy repairs was 32W/cm\textsuperscript{2}, resulting in a mean bursting pressure of 229mmHg. It is noticeable from these results that as the power density is increased from 32W/cm\textsuperscript{2} to 39W/cm\textsuperscript{2} the mean bursting pressure is significantly reduced from 228.7mmHg to 42.6mmHg. This dramatic reduction in arteriotomy strength is as a result of the high
incidence of tissue carbonisation along the length of the vessel. Tissue fusion became extremely difficult as the laser power increased towards 0.3W. It should be observed that the maximum bursting pressure obtained during these experiments was 453mmHg.

4.3.2. **Variation in the composition of bovine albumin.**

The following two sections summarise the results obtained from bursting pressure tests on longitudinal arteriotomies using a fixed laser power that gave the optimum burst pressure results. However, in these experiments the concentration of the bovine albumin used in the laser-activated glues was varied.

4.3.2.1. **Argon ion laser. With bovine albumin. No chromophore.**

The results of this category are shown in table 4.5.

<table>
<thead>
<tr>
<th>[Bovine albumin]</th>
<th>P</th>
<th>Power density</th>
<th>Mean bursting pressure</th>
<th>Range of bursting pressures</th>
</tr>
</thead>
<tbody>
<tr>
<td>% w/w</td>
<td>watts</td>
<td>watts/cm²</td>
<td>mmHg</td>
<td>mmHg</td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
<td>25</td>
<td>23.8</td>
<td>21-28</td>
</tr>
<tr>
<td>40</td>
<td>0.2</td>
<td>25</td>
<td>101.4</td>
<td>45-267</td>
</tr>
<tr>
<td>50</td>
<td>0.2</td>
<td>25</td>
<td>87</td>
<td>38-140</td>
</tr>
<tr>
<td>60</td>
<td>0.2</td>
<td>25</td>
<td>44</td>
<td>38-60</td>
</tr>
<tr>
<td>70</td>
<td>0.2</td>
<td>25</td>
<td>70</td>
<td>51-87</td>
</tr>
</tbody>
</table>

Table 4.5. Table showing the burst pressures obtained for the arteriotomies for the vessels repaired using different bovine albumin concentrations.

In this experiment irradiation durations of 20 seconds per spot were used to repair the arteriotomies. No coagulation of blood vessel proteins was observed, although surface drying of the vessel edges and albumin were apparent. This suggests that the temperature of the blood vessel was not sufficient to cause coagulation of tissue proteins because the albumin has a minimal absorption at the Argon ion laser wavelengths. As the bovine albumin dries during irradiation it forms a seal around the cut edges. Although the arteriotomy was strengthened by the drying of the bovine albumin, this is not a result of tissue coagulation. The optimum bovine albumin
concentration used in this set of artery repairs was 40% w/w, resulting in a mean bursting pressure of 101mmHg. It appears that as the concentration of bovine albumin increases beyond 40% w/w, the resulting strength of the repaired vessels is reduced. Although solutions with higher concentrations of bovine albumin appeared more suitable as tissue glues, increased viscosity did not facilitate manipulation of the glue solutions into and around the longitudinal slits. The reduction in vessel strength may be due to the lack of albumin between the opposed vessel edges.

4.3.2.2. Argon ion laser with bovine albumin and chromophore.

The results from this group of vessel repairs are shown in table 4.6. At each bovine albumin concentration the solutions were combined with the same chromophore, Basic fuchsin.

<table>
<thead>
<tr>
<th>[Bovine albumin]</th>
<th>P</th>
<th>Power density</th>
<th>Mean bursting pressure</th>
<th>Range of bursting pressures</th>
</tr>
</thead>
<tbody>
<tr>
<td>% w/w</td>
<td>watts</td>
<td>watts/cm²</td>
<td>mmHg</td>
<td>mmHg</td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
<td>25</td>
<td>224.1</td>
<td>144-304</td>
</tr>
<tr>
<td>40</td>
<td>0.2</td>
<td>25</td>
<td>254.4</td>
<td>124-350</td>
</tr>
<tr>
<td>50</td>
<td>0.2</td>
<td>25</td>
<td>214.5</td>
<td>72-497</td>
</tr>
<tr>
<td>60</td>
<td>0.2</td>
<td>25</td>
<td>222.3</td>
<td>87-237</td>
</tr>
<tr>
<td>70</td>
<td>0.2</td>
<td>25</td>
<td>218.8</td>
<td>91-330</td>
</tr>
</tbody>
</table>

Table 4.6. Table showing the burst pressure obtained for the arteriotomies repaired using bovine albumin (varying concentration) and Basic fuchsin.

In accordance with the earlier experiments where longitudinal arteriotomies were repaired with Argon ion laser and a laser-activated glue with fixed composition of bovine albumin (40%w/w) (see section 4.3.1.3), the results of the current vessel repairs are encouraging. The mean bursting pressures of the arteriotomies repaired using the five different bovine albumin compositions are all in excess of 200mmHg. The highest observed pressure being 497mmHg, well in excess of suprasystolic pressure. This investigation was limited to a laser power of 0.2W. This rating as chosen because in previous experiments (see section 4.3.1.3) arteries repaired using this intensity were the strongest, without resulting in carbonisation to the surface of
the blood vessel. In all instances irradiating was continued until a recognisable endpoint was observed. This effect was noticeable after approximately 5 seconds. In the majority of cases carbonisation of the tissue was kept to a minimum. In contrast to previous experiments, the concentration of bovine albumin in the laser-activated glue was not found to be a significant contributor to vessel strength. This particular preparation does not rely on the ability of the bovine albumin to move into areas between the cut edges, but more on the presence of chromophore, enhancing the absorption of laser energy.

4.3.3. Variation in the chromophore.

The chromophores chosen for combination with bovine albumin (40% w/w) were Eosin Y (Ey), Erythrosin (E), Phloxin (P) and Basic fuchsin (BF) (see section 4.2.2 for details). The results from this group of welds are shown in table 4.7.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Power</th>
<th>Power density</th>
<th>Irradiation duration</th>
<th>Mean bursting pressure</th>
<th>Range of bursting pressures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>watts</td>
<td>watt/cm²</td>
<td>sec/spot</td>
<td>mmHg</td>
<td>mmHg</td>
</tr>
<tr>
<td>Ey</td>
<td>0.2</td>
<td>25</td>
<td>10</td>
<td>195</td>
<td>120-439</td>
</tr>
<tr>
<td>E</td>
<td>0.2</td>
<td>25</td>
<td>5</td>
<td>129</td>
<td>31-355</td>
</tr>
<tr>
<td>P</td>
<td>0.2</td>
<td>25</td>
<td>10</td>
<td>254</td>
<td>23-343</td>
</tr>
<tr>
<td>BF</td>
<td>0.2</td>
<td>25</td>
<td>5</td>
<td>218</td>
<td>68-427</td>
</tr>
</tbody>
</table>

Table 4.7. Table showing the burst pressure obtained for the arteriotomies repaired using bovine albumin (40%w/w) with chromophore.

For each chromophore group, lasing of the tissue was continued until a recognisable end-point as achieved. This manifested itself in different ways depending on the chromophore in the activated glue. For Eosin Y and Phloxin, dyes with relatively high quantum yields of fluorescence, 0.23 and 0.20 respectively (see chapter 3 for details), the typical end-point was masked by the fluorescence of the dyes on the surface of the blood vessel. As a result it was difficult to distinguish between the glowing of the tissue due to blood vessel coagulation, and the glowing due to fluorescence of the Eosin Y and Phloxin laser-activated glues. Conversely, Erythrosin
and Basic fuchsin have lower quantum yields of fluorescence, 0.021 and <0.05 respectively. As a consequence, when such laser-activated glues were irradiated, the recognisable end-point was observed. In conclusion, the use of these chromophores helps to prevent over-irradiation, thus resulting in stronger blood vessels. It is noticeable that each laser-activated glue requires a different irradiation duration. This is determined by the fluorescence yield. Dyes with the lower fluorescence yields required shorter exposure times (Erythrosin and Basic fuchsin, 5 sec/spot) in comparison to Eosin Y and Phloxin, where exposure times of 10 sec/spot were necessary for tissue coagulation. The results achieved in this set of experiments were good, with vessel strengths in the region of 200mmHg. The maximum burst pressure attainable was 439mmHg for arteriotomies repaired using Eosin Y-activated glues.

4.4. Discussion.

The initial in vitro bursting pressure studies were carried out with the following aims.

- To gain experience of laser-welding using a simple model such as the repair of longitudinal arteriotomes.
- To determine the optimum technique for laser welding, by studying the bursting pressure as a function of incident laser power and irradiation duration.
- To assess the possibility of using externally applied laser-activated glues consisting of a suitably absorbing chromophore and addition protein material.

Following bursting pressure studies on a large sample (in excess of 240) the following general points were observed:

- The physical quality of porcine splenic arteries declined following collection from the abattoir. Experience showed that the weld strength of repairs in old vessels (>5 days following collection) were significantly weaker than weld strengths found in vessels used immediately after collection.
- During the gathering and preparation of the porcine splenic arteries the adventia was completely removed. This may affect the bursting strength of the repair arteriotomy.
• Weld strength was affected by moisture around the vessel edges. In all instances the arteriotomies were dried thoroughly prior to irradiation.

• Apposition of the cut vessel edges was critical to the success of laser repair. Eversion of the arteriotomy, producing a distinct area of vessel overlap, was essential to the success.

• Irradiation was applied as a series of approximately 10 spot welds along the length of the arteriotomy. Aiming of the laser beam along the everted edges was essential, firstly to ensure successful tissue coagulation, and secondly to avoid carbonisation of the surrounding area, which would affect the overall vessel strength.

Throughout the arteriotomy repairs the size of the laser spot was 1mm. This diameter was chosen as it best suited the repair of porcine splenic arteries whose diameters were in the range 1-2mm. As the spot size was increased beyond 1mm, it was found the resulting welds were weak because the temperatures at the irradiation site were insufficient to cause blood vessel coagulation. Conversely, it was not practical to use spot sizes below 1mm, since irradiation caused rapid tissue carbonisation and extensive thermal damage, weakening the repaired vessel.

The onset of visual changes to the surface of the irradiated vessel was used as an indication for completion of irradiation. In certain categories, particularly the non-chromophore groups, these visible changes were not evident and tissue bonding was not achieved. When the Argon ion laser was used with the chromophore surface glowing was observed, demonstrating that protein coagulation had been achieved. The only problem experienced with recognition of a suitable end-point occurred when using chromophores with high fluorescence yields.

Section 4.3.1 showed the importance of the rate of input of laser energy with regards the quality of bonding. For arteriotomies repaired using a) no additive and b) with bovine albumin, the mechanical strengths were insignificant. Although irradiation durations in excess of 20 sec/spot were used with a power density of 39W/cm², the maximum burst pressures were 80 and 37mmHg respectively. The results of these bursting pressure studies cast doubt on the success of vessel repair without a suitably
absorbing chromophore. Longer irradiating durations (>20 sec/spot) may result in tissue fusion, but other factors have to be considered.

1. The use of such long irradiation durations results in the dissipation of heat into areas surrounding the arteriotomy. This leads to drying and shrinkage of the tissue.

2. It would be difficult to expect the operator to maintain the position of the laser beam over the desired position for several minutes.

3. The primary objective for developing laser assisted anastomosis is to reduce the operative time. Laser repairs requiring durations in excess of 15/20 minutes would be unacceptable.

For vessels repaired with the application of chromophore, the optimum bursting pressures were obtained at laser powers of 0.2 and 0.25W. Below these powers, consistent welds were not obtained because the temperature required for bonding had not been achieved. The exposure time for irradiating with these laser powers was 5 sec/spot. This was a considerably shorter time than that required for bonding without chromophore, and gave significantly better weld strengths. Shorter irradiation durations restricted the incidence of thermal damage to small volumes around the anastomosis. At power levels in excess of 0.25W the rate of temperature rise was high and caused carbonisation and widespread thermal damage almost immediately following irradiation. This resulted in a drop in mechanical strength of the bond. Comparing the optimum results from each irradiating condition, it was obvious that the laser activated glue repairs were consistently stronger than vessels repaired using the two other irradiating conditions, and that the repairs could withstand suprasystolic pressures. The second set of vessel repairs was used to assess the quality of laser welding using different concentrations of protein material with and without chromophore. Bovine albumin was selected as a potential tissue glue, as a result of previous experiments where several protein materials (ovalbumin, collagen, human albumin) were tested for their ease of coagulation, and strength of welds produced in glass slides and cardiac catheters.
Arteriotomies repaired using a laser glue consisting of bovine albumin of varying concentrations, gave marginally better results than vessels repaired using standard laser assisted vascular anastomosis techniques. Throughout the repair of this set of welds there was never any evidence to suggest that coagulation was taking place. This was reflected in the bursting pressure results. The optimum bovine albumin concentration was 40% w/w giving a mean bursting pressure of 101mmHg. Increasing the bovine albumin concentration beyond 40% w/w resulted in a reduction in the mean bursting pressure.

Arteriotomies repaired with the addition of bovine albumin and chromophore produced significantly stronger welds than those without chromophore. Unlike the previous repairs (see section 4.3.1.2), bursting pressure strengths of these welds showed no significant variation as the albumin concentration was altered. In comparison to the 'no chromophore' category, these repairs showed characteristic signs of coagulation. The optimum bovine albumin concentration in this group of laser activated glues was 40% w/w, with a mean bursting pressure of 254mmHg. However, the standard deviation in bursting pressure was only 14mmHg for the range of albumin concentrations 20-70% w/w, and all were in excess of 200mmHg. This indicated that the albumin concentration was not the determining factor, and that the presence of chromophore was of greater importance.

The final category of vessel repairs were assessed using four different chromophores to prepare laser-activated glues. Rather than limiting the vessel repair to Basic fuchsin laser-activated glues, which may not be suitable for use in vivo, three other dyes were chosen. The additional chromophores showed no history of adverse effects such as carcinogenicity or mutagenicity. Phloxin laser-activated glues resulted in the strongest vessel repairs, with a mean bursting pressure of 254mmHg. The highest attainable bursting pressure was 439mmHg. With the exception of Erythrosin activated glues, the mean bursting pressures were in the region of 200mmHg.

A number of repaired arteriotomies gave lower than expected burst pressure results, even when repaired using the 'optimum' power ratings and irradiation durations. These results are a cause for concern and are a consequence of.
Arteries have different physical features, with varying thickness and diameter. The inherent differences in an artery structure make consistent bursting strength unlikely.

Difficulty in ensuring complete drying and removal of blood from the vessel edges, in addition to maintaining vessel overlap affects the bond quality.

The arteriotomy repairs were strength tested in vitro using Ringers solution. Invariably, the arteriotomies leaked from tiny pinholes around the sutures. This is in stark contrast to the in vivo environment where blood would clot and seal these pinhole faults.

However, there were a number of vessels that failed unexpectedly. The mean bursting pressures of arteriotomies repaired using the optimum conditions were above normal physiological requirements. The use of chromophores to increase the absorption of Argon laser radiation for application to laser tissue welding has been reported in several papers. Chuck et al. used Fluorescein isothiocyanate with Argon ion laser (0.1-0.3W) to perform longitudinal abdominal aortotomies (7mm) in rabbits. Bursting pressures (n=7) of 164mmHg with chromophore and 147mmHg without chromophore were reported. There is a significant difference between the strengths of the ‘no chromophore’ repair reported by Chuck, and those presented in this chapter (147mmHg compared with <40mmHg). This difference is explained by the fact that significantly higher irradiation durations per spot were used by Chuck. The difference was not so large for the ‘chromophore’ category of repairs. Brooks repaired longitudinal arteriotomies (4mm) of porcine coronary arteries in vitro using Basic fuchsin as a chromophore. Mean bursting pressures of 249mmHg (standard deviation 83mmHg) at 40W/cm² were recorded without chromophore (standard laser welding techniques) and 282mmHg (standard deviation 63mmHg) at 5.6W/cm² with chromophore, but no laser adhesive. The use of chromophore only marginally increased the quality of repair. The arteriotomies were repaired using irradiation durations ranging from 20 to 60 seconds even when chromophore was used. The conditions used in Brooks study are not comparable to the exposure times used to repair vessels in this chapter. Such long durations completely undermine the
technique as a possible replacement for conventional suturing. More recently, investigators have begun to use ‘laser glues’ in laser welding. Poppas et al [94] performed end-to-end anastomosis of canine ureters in vitro using a Krypton ion laser (532nm) and a laser tissue glue consisting of 40% human albumin and Fluorescein. Mean bursting pressures of 9mmHg (standard deviation 4mmHg) at 7.14W/cm² were recorded without chromophore and 281mmHg (standard deviation 24mmHg) at the same power density with chromophore. Although comparison between the results of Poppas et al [94] and those documented in this chapter may not be applicable because of differences in surgical models, and technical parameters such as laser power and mode of irradiation, there were similarities in the overall conclusions of the two studies.

In conclusion, the results of this chapter have shown that the Argon laser with activated glue can be used to repair longitudinal arteriotomies in vitro with sufficient strength to withstand typical blood pressures found in the body. The addition of chromophores to enhance absorption of laser light was essential to the formation of a suitable weld in a reasonable time. The formation of acceptable welds have been achieved using markedly lower laser powers and irradiation durations compared to previous reports [59,60,61]. The results of any reduction in thermal damage to the blood vessel proteins using these conditions will be investigated in proceeding chapters.
5. **BURSTING PRESSURE STUDY II. END-TO-END ANASTOMOSIS OF PORCINE SPLENIC ARTERIES.**

5.1. **Introduction.**

In the preceding chapter it was shown that longitudinal arteriotomies could be repaired using an Argon ion laser in combination with a laser activated glue consisting of additional protein material and chromophore. Bursting pressure studies indicated that arteries repaired at the ‘optimum’ power could withstand suprasystolic intraluminal pressures. The repair of longitudinal arteriotomies was selected as the initial testing criteria as it was a straightforward procedure, and was thought the most suitable method for familiarisation with the use of lasers for vascular repair. However, arteriotomy repair is not the most valid test for application of laser welding *in vivo*. For this reason the mechanical strengths of end-to-end anastomoses were studied. The preparation of such anastomoses require a greater degree of surgical skill than simple arteriotomies, but have more significance for *in vivo* testing.

This *in vitro* study was undertaken to assess the quality and strength of end-to-end anastomoses repaired using Argon laser with laser activated glues as a reinforcement to bonding. As in the previous chapter, bovine albumin was used as the protein material. In addition to using bovine albumin as the ‘laser glue’, a number of end-to-end anastomoses were repaired with and without the addition of a further protein, bovine fibrin. Bovine fibrin is a readily available protein and has been used as a tissue glue in numerous surgical applications, including neurosurgical, ear nose and throat and gastrointestinal surgery for anastomosis of nerves, blood vessels and biliary ducts [112]. Kamiji et al [115] performed microvascular anastomosis on rat femoral arteries and veins using bovine fibrin glue and a soluble intravascular stent made of polyethylene glycol. The fibrin glue consisted of: 1g of bovine fibrin dissolved in 10ml of sterilised water, 5000 units of human thrombin dissolved in 1ml of 0.5M Calcium Chloride, 7ml of aprotinin and 4ml of sterile water. A 100% patency rate was reported, and the anastomoses were strong enough to withstand pressures of 250mmH$_2$O. Human
Chapter Five: Bursting pressure study II. End-to-end anastomosis of porcine splenic arteries.

Fibrin has been used extensively as a tissue glue. Silverstein and Mellinger \cite{16} undertook a study to evaluate the use of fibrin glue for vasovasostomy, and to compare the results using this technique with conventional sutured vasovasostomy. The fibrin glue consisted of human fibrinogen, aprotinin, dried thrombin and calcium chloride. The fibrin glue technique of vasal anastomosis gave a patency rate comparable to the sutured technique, required less operative time and required less microsurgical skill. Ball et al \cite{113} investigated the reanastomosis of rats vas deferens, comparing i) suture only, ii) carbon dioxide laser assisted, and iii) fibrin based tissue adhesive. The fibrin based adhesive consisted of human fibrin, factor VIII and calcium with a thrombin solution. Evaluation of the anastomosis following repair revealed comparable results between the three surgical groups. The fibrin adhesive repair gave the highest patency rate 89%. The laser-assisted and suture repair groups resulted in considerably longer operative times (39 and 46 minutes respectively) compared to fibrin based repairs. The fibrin sealants \cite{15,16,113} used in these and other studies used a multicomponent biological adhesive. They involve the use of factors resulting in coagulation, and produce a fibrin seal around the anastomosis. Fibrinogen, when combined with thrombin and calcium is converted to the fibrin monomer. Upon the addition of clotting agents such as factor 13, and calcium, the fibrin monomer undergoes crosslinking and a stable fibrin polymer is produced. Aprotinin is added to slow the degradation of fibrin. In a comparative study \cite{114}, the effect of six different compositions of fibrin based glues were assessed for their mechanical strength. Standard dorsal skin incisions in Wistar rats were prepared and various compositions of fibrinogen, thrombin and factor XIII were used to repair the wound. Eight days following surgery the animals were sacrificed and the wounds were excised. Strips of these wounds were mechanically tested, and the stress, strain, elasticity and work required to rupture the wounds was calculated.

In this study mechanical bursting pressure tests were performed on the anastomosis immediately after completion of welding, and a number of anastomoses were retained for histological examination.
5.2. **Materials and methods.**

Porcine splenic arteries were collected and prepared as described in section 4.2. End-to-end anastomoses were carried out on short lengths of artery, (up to 5cm), dissected transversely. The separated vessel was supported on a fine stainless steel high pressure gas tube, and the vessel edges were drawn together with two horizontal mattress sutures (Surgibond 5/0). The anastomotic repairs were fashioned as a series of 'spot welds', typically each anastomosis required 10-15 spots. Final preparation of the anastomosis and irradiation continued as described in earlier sections. In accordance with the repair of longitudinal arteriotomies, the end-point for lasing was determined by visual effects corresponding to protein coagulation\cite{39}. A range of laser powers were used to give the optimum bursting strength, while minimising the extent of thermal damage.

5.2.1. **Bovine albumin.**

As a result of previous investigations into the dependence of bovine albumin concentration on arteriotomy strength (section 4.3.2), a bovine albumin concentration of 40% w/w was used. Preparation of this solution has been described in an earlier chapter.

5.2.2. **Chromophore.**

Two different chromophores were used in combination with bovine albumin to create laser-activated glues, Basic fuchsin and Eosin Y. The concentration of dye was adjusted to ensure comparable absorbances amongst the different albumin/chromophore mixtures.

5.2.3. **Measurement of bursting pressure.**

Immediately following repair, each anastomosis was subjected to a series of bursting pressure tests. The bursting pressure apparatus and procedures have been described in a previous chapter (see section 4.2.3, page 124).
5.3. **Results.**

The results of the three bursting pressure studies are presented below.

5.3.1. **Argon ion laser. With bovine albumin, no chromophore.**

Table 5.1 shows the results of the mechanical tests of the end-to-end anastomoses repaired at four different power settings.

<table>
<thead>
<tr>
<th>P</th>
<th>Power density</th>
<th>Irradiation duration</th>
<th>Mean bursting pressure (n=10)</th>
<th>Range of bursting pressures</th>
</tr>
</thead>
<tbody>
<tr>
<td>watts</td>
<td>watts/cm²</td>
<td>sec/spot</td>
<td>mmHg</td>
<td>mmHg</td>
</tr>
<tr>
<td>0.15</td>
<td>19</td>
<td>20</td>
<td>31.6</td>
<td>23-40</td>
</tr>
<tr>
<td>0.2</td>
<td>26</td>
<td>20</td>
<td>38.6</td>
<td>18-87</td>
</tr>
<tr>
<td>0.25</td>
<td>32</td>
<td>20</td>
<td>50.7</td>
<td>37-62</td>
</tr>
<tr>
<td>0.3</td>
<td>38</td>
<td>20</td>
<td>46.7</td>
<td>25-61</td>
</tr>
</tbody>
</table>

Table 5.1. Table showing the burst pressures obtained for end-to-end anastomoses repaired with bovine albumin (40%w/w).

A visual end-point could not be used as an indicator for the termination of lasing because the power densities and irradiation durations were insufficient to cause protein coagulation. The optimum power density for end-to-end anastomosis in this set of irradiating conditions was 32W/cm² giving a mean bursting pressure of 51mmHg. The maximum attainable bursting pressure was 87mmHg, however, none of the repairs resulted in anastomoses able to withstand pressures within the normal physiological range.

For repairs fashioned irradiating at a power of 0.2 watts there was evidence of some minor thermal injury on one side of the tissue. Presumably this was because of overlapping. The vessel was not glued and the special stain for elastin and collagen elements failed to show that these tissue components were glued. Upon examination of a vessel repaired at a power of 0.3 watts a small crater was noted at the point of laser application. This artery was unhealthy, in that it displayed signs of intimal hyperplasia, which was rather unfortunate because the elastic Van Gieson section suggested that the tissue might have been glued.
5.3.2. Argon ion laser. With bovine albumin and Basic fuchsin.

The results of this category are shown in table 5.2. End-to-end anastomosis were performed using a series of four laser powers in the presence of bovine albumin 40% w/w, and Basic fuchsin (0.02mol/dm³).

<table>
<thead>
<tr>
<th>P</th>
<th>Power density</th>
<th>Mean irradiation duration to end point</th>
<th>Mean bursting pressure (n=10)</th>
<th>Range of bursting pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>watts</td>
<td>watts/cm²</td>
<td>sec/spot</td>
<td>mmHg</td>
<td>mmHg</td>
</tr>
<tr>
<td>0.15</td>
<td>19</td>
<td>10</td>
<td>313.8</td>
<td>92-523</td>
</tr>
<tr>
<td>0.2</td>
<td>26</td>
<td>5</td>
<td>345.7</td>
<td>82-558</td>
</tr>
<tr>
<td>0.25</td>
<td>32</td>
<td>5</td>
<td>525.5</td>
<td>411-655</td>
</tr>
<tr>
<td>0.3</td>
<td>38</td>
<td>&lt;5</td>
<td>453</td>
<td>302-719</td>
</tr>
</tbody>
</table>

Table 5.2. Table showing the burst pressures obtained for end-to-end anastomoses repaired with a bovine albumin (40%w/w) and Basic fuchsin laser activated glue.

The presence of chromophore within solutions of bovine albumin greatly enhanced the absorption of laser radiation. As a consequence the heat developed at the irradiation site was sufficient to cause thermal alteration to the protein structure. Depending on power density, lasing was continued for a range of exposure times until an end-point was observed. Unlike previous investigations earlier in this thesis where I reported that the presence of chromophore ‘obscured’ the appearance of visual changes on the surface of the blood vessel (section 4.3.3), Basic fuchsin did not impair the assessment of the end-point. The optimum power density in this category of repair was 32W/cm² with an average bursting pressure of 526mmHg, this compares with 50.7mmHg for the ‘no chromophore’ group, a tenfold increase in mechanical strength. All mean bursting pressures were consistently above the normal physiological range. In a number of samples, particularly using powers of 0.2 and 0.25W, the blood vessels did not burst from the glued anastomotic site, however, strength testing stopped prematurely because of leaks from sutures and side branches.

143
Two different samples were examined histologically, both vessels had been subjected to repair using a power of 0.2 watts. One sample had been dissected longitudinally, the other prepared transversely. Examination of the longitudinal section revealed that the tunica media was slightly separated from the tunica intima and there was some glue in the intima. As expected the media seemed to be undamaged by the laser, however the elastic Van Gieson stain showed physical disruption of the tissue. Macroscopic examination of the transverse section showed signs of glue in the lumen, the glue had coagulated 3-4 strands of the media. Microscopically the elastic Van Gieson stain showed that collagen appeared to be unaffected by the treatment.

5.3.3. Argon ion laser. With bovine albumin and Eosin Y.

The mechanical bursting pressure results are given in table 5.3. End-to-end anastomoses were performed using the same conditions as described in the previous section. Rather than using Basic fuchsin, Eosin Y (0.017mol/dm³) was added to solutions of bovine albumin 40% w/w.

<table>
<thead>
<tr>
<th>P</th>
<th>Power density</th>
<th>Mean irradiation</th>
<th>Mean bursting pressure</th>
<th>Range of bursting pressures</th>
</tr>
</thead>
<tbody>
<tr>
<td>watts</td>
<td>watts/cm²</td>
<td>duration to end</td>
<td>(n=10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>point</td>
<td>mmHg</td>
<td>mmHg</td>
</tr>
<tr>
<td>0.15</td>
<td>19</td>
<td>10</td>
<td>382.1</td>
<td>328-425</td>
</tr>
<tr>
<td>0.2</td>
<td>26</td>
<td>&lt;5</td>
<td>366.6</td>
<td>168-477</td>
</tr>
<tr>
<td>0.25</td>
<td>32</td>
<td>~3</td>
<td>227.0</td>
<td>95-329</td>
</tr>
<tr>
<td>0.3</td>
<td>38</td>
<td>1</td>
<td>226.8</td>
<td>118-442</td>
</tr>
</tbody>
</table>

Table 5.3. Table showing the burst pressure obtained for end-to-end anastomoses repaired with a bovine albumin (40%w/w) and Eosin Y laser activated glue.

In accordance with the last category of vessel repair, the presence of Eosin Y in the laser activated glue greatly increased the absorption of Argon laser radiation. In fact, the absorbance of the Eosin solution at the Argon emission wavelengths slightly exceeded that of the Basic fuchsin activated glue. As a consequence there was a
difference in exposure times between the two groups. The major disadvantage of this particular laser glue was the difficulty experienced in estimating the onset of blood vessel coagulation. The usual intense glow from the tissue surface was masked by the fluorescence of Eosin Y. This severely impaired the ability to assess the termination of lasing. The optimum power density in this category was 19W/cm², giving a mean bursting pressure of 382.1mmHg, compared with 51mmHg for the ‘no chromophore’ group. A sevenfold increase in mechanical strength. Although the welds repaired using Eosin Y based laser-activated glues where not as strong as those prepared using Basic fuchsin, all welds gave mean bursting pressures in excess of the physiological range. Vessels subject to repair using laser powers of 0.2 watts and 0.3 watts were examined histologically. The anastomosis effected at 0.2 watts was lightly glued together, although there was slight thermal injury. Macroscopically the anastomoses prepared at the higher laser power showed evidence of surface charring. Microscopically the char showed as a lump of glue, and the two edges of the tissue were not united. One possibility is that the glue seeped in between the vessels, since the cut edges seemed to be sealed. An elastic Van Gieson stained section showed glue in the media.

5.4. Discussion.

In continuation from the longitudinal arteriotomy repair experiments, the primary objective of this study was to explore the possibility of using a coagulatable protein, bovine albumin, in combination with a suitable absorbing chromophore, to prepare end-to-end anastomosis in vitro. The use of Basic fuchsin and Eosin Y with bovine albumin increased the consistency of vessel welds and resulted in mean bursting pressures well above suprasystolic pressures. The bursting strength results of Basic fuchsin mixtures were marginally better than those obtained using Eosin Y (525.5mmHg standard deviation (s.d)97mmHg, and 382mmHg s.d 35mmHg respectively). This is explained by the high incidence of surface thermal damage observed in anastomosis repaired using the
latter. Examination of Eosin Y treated welds following irradiation indicated a high degree of carbonisation on the surface of the anastomoses, see figure 5.1. However, histological examination of a number of anastomoses revealed that the extent of thermal damage was limited, and that it did not penetrate deeply into the media. Charring of the splenic arteries was evident when laser powers as low as 0.2W were used. Anastomoses repaired using power densities in excess of 26W/cm² caused significant problems with respect to controlling the tissue temperature and the onset of vascular carbonisation. Operating times as low as 1-2 sec/spot were commonplace. Such short exposure times reduced the degree of operator control, resulting in extensive thermal damage. A further disadvantage of Eosin Y activated glue solutions, was the difficulty in assessing a suitable end-point for irradiating. This invariably resulted in the over irradiation of the anastomoses. The usual intense back scatter from the tissue surface was disguised by the high fluorescence of Eosin during irradiation.

In conclusion, the results of this section have indicated that the Argon laser in combination with mixtures of bovine albumin and chromophore can be used to perform end-to-end anastomoses, with sufficient strength to withstand blood pressures found in the body. The bursting strength studies confirmed the results of previous investigations, that the addition of a chromophore to enhance the absorption of laser light was essential in the formation of welds strong enough to withstand typical physiological requirements.
Figure 5.1: Photograph of an artery repaired using Eosin Y laser activated glue using a power of 0.25W, evidence of thermal damage.
5.5. The *in vitro* reinforcement of end-to-end anastomosis using bovine serum albumin and bovine fibrin.

5.5.1. Introduction.
A number of studies\(^{115,116,117}\) have shown that fibrin sealant contributes favourably to wound healing by producing local haemostasis and by encouraging the influx of macrophages that are able to produce factors causing angiogenesis, fibroblast proliferation and collagen production. The aim of this study was to investigate the use of bovine fibrin (combined with mixtures of bovine albumin and chromophore) as a possible laser activated glue for the reinforcement of bonding. Mechanical bursting pressure results of reinforced anastomoses were compared to those repaired using standard welding techniques without the addition of any external laser activated glues. A number of repaired vessels were retained for histological examination.

5.5.2. Materials and methods.
Porcine splenic arteries were collected and prepared as described in section 4.2. End-to-end anastomoses were performed as outlined in section 5.2. In each case where applicable, irradiating was continued until a glowing of the tissue surface was noticeable. As a result of previous investigations into the dependence of weld strength as a function of incident laser power, only two laser powers were used for this investigation, 0.2 and 0.25W. These laser powers gave the 'optimum' weld strength for repairs using laser activated glues. A spot size of 1mm was used during these experiments.

Three different coagulant solutions were assessed:
- No additive.
- With bovine albumin and chromophore.
- With bovine albumin, bovine fibrin and chromophore.
5.5.2.1 **Bovine fibrin and albumin solutions.**
Powdered bovine fibrin (Aldrich Chemical Company Ltd, Poole, UK) was used to prepare a solution with a concentration of \( \approx 5\% \) w/w. The powdered fibrin was added slowly to a stirred solution of 1M Sodium hydroxide. As a result of previous experiments (see section 4.3.2), when weld strength was studied as a function of bovine albumin concentration with and without albumin, a bovine albumin concentration of 40\% w/w was used. Bovine albumin solutions were prepared as described in section 4.2.1.

5.5.2.2. **Chromophore.**
Eosin Y was used in combination with bovine albumin and/or bovine fibrin to create laser activated glues, (see chapter 3 for details). The concentration of Eosin Y was comparable to those of previous laser activated glues, \( 4 \times 10^{-2} \) mol/dm\(^3\).

5.5.2.3. **Measurement of bursting pressure.**
Following laser repair each anastomosis was subjected to a series of bursting pressure tests. The bursting pressure apparatus and procedure have previously been described in section 4.2.3.

5.5.3. **Results.**
The results of bursting pressure studies on the three coagulant solutions are presented in the proceeding sections.

5.5.3.1. **Argon ion laser. No bovine albumin or chromophore.**
The results from this group of end-to-end anastomosis are shown in table 5.4.
Chapter Five: Bursting pressure study II. End-to-end anastomosis of porcine splenic arteries.

As with previous experiments, (both longitudinal and end-to-end anastomoses (sections 4.3.1.1 and 5.3.1)), few successful repairs were produced using the Argon ion laser on its own. At no stage during irradiation was there any evidence of visual changes to the irradiated vessel. In previous experience the absence of such visual changes indicated that the coagulation temperature had not been achieved, and that the combination of power density and irradiation duration were insufficient to cause tissue fusion. The only visible change noticeable in this group of end-to-end anastomoses was a drying of the vessel edges. The maximum burst pressure obtained using the two different laser power densities was 87mmHg.

5.5.3.2. Argon ion laser with bovine albumin and Eosin Y.

The results from this group of vessel repairs are shown in table 5.5. The laser activated glue consisted of bovine albumin 40% w/w and the chromophore, Eosin Y (0.04mol/dm³).

<table>
<thead>
<tr>
<th>P</th>
<th>Power density</th>
<th>Irradiation duration</th>
<th>Mean bursting pressure (n=10)</th>
<th>Range of bursting pressures</th>
</tr>
</thead>
<tbody>
<tr>
<td>watts</td>
<td>watts/cm²</td>
<td>sec/spot</td>
<td>mmHg</td>
<td>mmHg</td>
</tr>
<tr>
<td>0.2</td>
<td>26</td>
<td>&lt;5</td>
<td>377.2</td>
<td>168-512</td>
</tr>
<tr>
<td>0.25</td>
<td>32</td>
<td>2-3</td>
<td>300</td>
<td>95-460</td>
</tr>
</tbody>
</table>

Table 5.5. Table showing the burst pressures obtained for end-to-end anastomoses repaired with a bovine albumin (40%w/w) and Eosin Y laser activated glue.
Anastomoses repaired using this particular laser activated glue resulted in welds strong enough to withstand typical intraluminal pressures. For each laser power used in this experiment, irradiating was continued until a visible change in the tissue surface was observed. As previously reported, section 4.3.1.3, the end-point was distinguishable as an intense glowing of the tissue surface, corresponding to coagulation of tissue proteins. For anastomoses repaired using a laser power of 0.2W the typical exposure time was approximately 5 seconds/spot. The onset of tissue coagulation was easily controllable and developed over a number of seconds. There was no evidence of any tissue carbonisation following the repair of these anastomoses (see figure 5.2). Conversely, when a higher laser power was used, (0.25W), the irradiation duration was in the region of 2-3 seconds/spot. In such cases it became difficult to control the extent of tissue coagulation, which developed almost instantaneously. Anastomoses repaired using this higher laser power were prone to over-irradiation, and carbonisation of the tissue surface was common (see figure 5.1). Thermal damage resulted in a weakening of the anastomoses (0.25W, 2-3 sec/spot, mean b.p=300 mmHg). The optimum power density employed in this set of anastomoses was 26 W/cm², resulting in a mean bursting pressure of 377.2 mmHg. The maximum bursting pressure obtained during these experiments was 512 mmHg.
Figure 5.2: Photograph of an artery repaired using an eosin Y laser activated glue irradiated at a laser power of 0.2W, no evidence of any thermal damage.
5.5.3.3. **Argon ion laser with bovine albumin, bovine fibrin and Eosin Y.**

The results from this category of vessel repairs are shown in table 5.6. A bovine albumin concentration of 40% w/w was used, with a bovine fibrin concentration of ≥5% w/w and eosin Y as the chromophore.

<table>
<thead>
<tr>
<th>P</th>
<th>Power density</th>
<th>Mean irradiation duration to end point</th>
<th>Mean bursting pressure (n=10)</th>
<th>Range of bursting pressures</th>
</tr>
</thead>
<tbody>
<tr>
<td>watts</td>
<td>watts/cm²</td>
<td>sec/spot</td>
<td>mmHg</td>
<td>mmHg</td>
</tr>
<tr>
<td>0.2</td>
<td>26</td>
<td>5</td>
<td>425.4</td>
<td>276-582</td>
</tr>
<tr>
<td>0.25</td>
<td>32</td>
<td>2-3</td>
<td>386</td>
<td>259-443</td>
</tr>
</tbody>
</table>

Table 5.6. Table showing the burst pressures obtained for end-to-end anastomoses repaired with a bovine albumin (40%w/w), bovine fibrin (≥5%w/w) and Eosin Y laser activated glue.

Anastomoses repaired using laser activated glues with the addition of bovine fibrin showed similar results to vessels repairs without bovine fibrin. The anastomoses were able to withstand typical supra-systolic pressures. Bovine fibrin activated glues produced more consistent anastomoses. All repairs were stronger than 250mmHg. As with previous experiments, irradiation was continued until there was a visible change to the tissue surface, corresponding to the coagulation of tissue proteins. Over-irradiation and carbonisation of the blood vessels was evident when irradiating at high laser powers. For laser powers of 0.25W, the typical irradiation duration was 2-3 seconds/spot, and tissue coagulation developed almost immediately. Invariably this resulted in widespread thermal damage, (see figure 5.3), and weakened the anastomoses (0.25W, 2-3 sec/spot Mean bp =386mmHg). Lasing using powers of 0.2W produced more consistent repairs, with a mean bursting pressure of 425mmHg. The onset of vessel coagulation was more controllable, developing over several seconds. The incidence of thermal damage was less frequent, (see figure 5.4). The ‘optimum’ power density employed in this set of anastomoses was 25.5W/cm², resulting in a mean bursting pressure of 425mmHg. The maximum attainable burst pressure using the bovine fibrin based laser-activated glue was 582mmHg.
Figure 5.3: Photograph of an artery repaired using an eosin Y/bovine fibrin laser glue at a power of 0.25W, evidence of thermal damage
Figure 5.4: Photograph of an artery repaired using an eosin Y/bovine fibrin laser glue at a power of 0.2W, no evidence of thermal damage.
5.5.4. **Discussion.**

The principle aim of the set of experiments was to assess the possibility of combining bovine fibrin with bovine albumin, and a suitably absorbing chromophore, to enhance the sealing of anastomoses. The repair of end-to-end anastomoses in porcine splenic arteries was assessed by bursting pressure. Reinforcement of bonds using bovine albumin (40% w/w) and chromophore significantly increased the mean bursting pressure to more than 300mmHg, almost a tenfold increase compared with the non-reinforced bonds. The difference in bursting pressure emphasised the need for suitably absorbing chromophores within the bovine albumin glue. As the laser power was increased from 0.2 to 0.25W there was a slight decrease in bursting pressure (377.2mmHg to 300mmHg respectively). This was as a result of over-irradiation, resulting in widespread carbonisation (see figure 5.1 compared with figure 5.2). Histological examination of anastomoses repaired using the bovine albumin/Eosin Y laser glue revealed a degree of thermal damage to the surface of the media, however the damage did not extend deep into the media. When solutions of bovine fibrin (≈5% w/w) were combined with bovine albumin the mean bursting pressure increased to values approaching 400mmHg. The increase in bursting strength was evident at both power densities. As with the ‘no fibrin’ anastomoses repairs, there was a decrease in weld strength as the laser power increased from 0.2 to 0.25W (425.4mmHg to 386mmHg respectively). Anastomoses repaired using the higher laser power displayed signs of vessel charring at the surface, (see figure 5.3 compared with 5.4). The resulting thermal damage resulted in a subsequent drop in weld strength. Histology revealed that some sections showed overlap, but all were glued successfully. The adhesion was between the cut edges of the very thick media. However, the external and internal elastic lamina were disrupted as a result of thermal injury. Elastic Van Gieson sections confirmed that the weld showed “sides-to-middle” overlap, and the adhesion was through the external elastic lamina.

A number of anastomoses repaired using ‘optimum’ power levels and exposure times ruptured at pressures below the expected thresholds. In the majority of cases, the
repairs leaked from small holes in the artery, either created by sutures, or due to side branch holes already present in the artery. The weld strengths were tested in vitro using Ringer’s solution. Such treatment is particularly harsh compared to that in vivo. Ringer’s has a tendency to ‘find’ faults in the anastomoses. Although a number of vessels failed prematurely, the mean bursting pressures of anastomoses repaired using the two laser activated glues were above normal physiological requirements. Conversely there were a number of welds that would not burst even as the pressure was increased beyond 500mmHg.

In conclusion, the results of this part of the experimental studies have shown that bovine fibrin can be added to solutions of bovine albumin laser activated glues without a detrimental affect on the bursting pressure. Indeed, the results indicate the presence of bovine fibrin increased the bursting strength of the repaired vessels. The results from this chapter reinforce the consideration that the addition of a chromophore to enhance the absorption of laser light was essential for the preparation of a patent weld.

A further section regarding the details of histological preparation and principles, including a summary of the overall histological findings is dealt with in chapter seven. The photographs referred to in this later chapter are used to illustrate whether sealing/bonding of the vessel edges has occurred, and to establish evidence of any thermal damage to the blood vessels.
6. THE PHOTOCHEMICAL FADING OF DYES- A POSSIBLE END-POINT FOR LASER ASSISTED VASCULAR ANASTOMOSIS.

6.1. Introduction.

The occurrence of visual changes during laser irradiation has been widely accepted by numerous research groups as an indication for the termination of welding. Although different visual changes have been reported, whether as a slight drying, shrinkage or colour change, the changes have been recognised as a suitable end-point for laser anastomosis.

Using an argon laser for the repair of longitudinal arteriotomies in the rat abdominal aorta, Vance reported the visual end-point as an increase in scattering of the laser corresponding to protein denaturation of the tissue, resulting in a bright glowing effect. Basu investigated the possibility of anastomosing the canine internal mammary artery and coronary artery using the carbon dioxide laser. Each anastomosis was slowly irradiated, trying to avoid burning of the blood vessels. The end-point used in this particular study was the onset of a slight brown discolouration of the tissue. In a comparative study to investigate the suitability of the Nd:YAG laser for tissue welding, end-to-end large bowel anastomoses were successfully created in rabbits. The irradiation was fashioned as an alternating 0.2 second on, 0.2 second off, using a laser power of 0.4 watts. A laser spot of 1mm was used to irradiate the anastomotic line. The appearance of tissue blanching, drying and contraction of the vessel edges were used for identification of the end-point.

The glowing of tissue during irradiation, corresponding to the denaturation of blood vessel proteins, typically occurs as the temperature of the tissue approaches 70°C. Earlier investigations into the bursting pressure strengths of end-to-end anastomosis repaired using three different irradiating conditions (see chapter 5), revealed that if the tissue surface is irradiated and the collagen denaturation is not reached (the case when vessels were repaired without the addition of suitably absorbing chromophore), an end-point cannot be distinguished and the bond strength is inconsistent. Alternatively, if irradiation is continued beyond 70°C, irreversible damage of the
Alternatively, if irradiation is continued beyond 70°C, irreversible damage of the tissue rapidly ensues, resulting in a reduced bond strength. Such circumstances were commonplace when irradiating anastomosis using chromophore activated glues, and laser powers higher than 0.2 watts. In these instances controlling the tissue temperature was difficult and the onset of a visible change was almost instantaneous. The disadvantage of basing the end-point determination upon a visual colour change is due to the fact that under typical conditions only a small area of tissue is irradiated (Spot size <2mm). Visible changes to such a small area are difficult to differentiate, particularly when the tissue is being irradiated, and the operator is wearing protective laser goggles, absorbing the potentially damaging laser radiation back scattered from the surface of the tissue. Photochemistry is an approach that could be used to reduce the uncertainty surrounding the determination of a suitable end-point. A coloured compound could be used that absorbed the argon laser radiation, generating heat before the denaturation temperature had been reached. However, once the denaturation temperature had been exceeded, the compound’s absorption characteristics are altered, reducing the absorption of the laser, leading to no further increase in temperature. Such a system would be ideal if the absorption change of the chromophore occurred after the absorption of a particular number of photons, corresponding to the denaturation temperature of blood vessel proteins. This particular approach has a two fold advantage. Firstly, there is a visual colour change signifying the end-point for irradiation, but more importantly, an absorption switch, whereby it is impossible to over-irradiate the tissue because the chromophore fades and no longer absorbs the laser radiation. Before considering a particular system it is important to consider a typical irradiation. This is of primary importance, as knowledge of the desired power rating and irradiation duration necessary to prepare a complete bond are required when selecting suitable systems yielding an end-point. Consider a typical irradiation, giving optimum results following previous bursting pressure studies. Laser power=0.2 watts
Irradiation duration = 5 seconds/spot.
Absorption = 0.90
Energy = Power × Irradiation duration × Absorption
Energy = 0.9 joules

\[ \text{Energy} = \frac{Nhc}{\lambda} \]

\[ \text{No of photons} = \frac{0.9 \times 488 \times 10^{-9}}{6.6 \times 10^{-34} \times 3 \times 10^{8}} \]

No of photons absorbed = 2.218 \times 10^{18}

Beam diameter = 1mm
Irradiation depth = 0.5mm
Volume of irradiation = 3.9 \times 10^{-4} \text{ cm}^3 \Rightarrow 3.9 \times 10^{-7} \text{ dm}^3

Using a laser glue with a dye concentration of 0.017 mol/dm³
No of molecules = Concentration of dye × Volume irradiated × \(N_A\)
No of molecules = 4.02 \times 10^{15}

It is evident from this rather simple calculation that there are a greater number of photons than molecules. Suggesting that if a photochemical approach was used, compounds with quantum yields of decolouration of the order \(4 \times 10^{15}/2 \times 10^{18}, 2 \times 10^{-3}\) would be desirable (if used with a similar concentration to the dye used in this calculation).

At the onset of this particular study, a number of different approaches yielding a suitable end point for laser welding were considered. Photochromic, thermochromic and the photoreduction of dyes.

6.1.1. **Photochromic approach.**

Photochromic compounds are materials that undergo reversible colour change when exposed to light of a particular wavelength. The photochromic properties of (E)-\(\alpha\)-(2,5-dimethyl-3-furyl-(ethylidine) isopropylidene succinic anhydride (Aberchrome
540), (chemical structure shown in figure 6.1), are due to photochemical ring opening and closure.

![Chemical Structure](image)

**Figure 6.1:** The photochemical ring closure of (E)-α-(2,5-dimethyl-3-furyl-(ethylidine)(isopropylidene)succinic anhydride.

Species B, the closed form, is a red coloured compound with a $\lambda_{\text{MAX}}$ of 496nm, is converted to species A (photobleached by photochemical ring opening) by the absorption of light of wavelength 500nm. The colour change of this particular compound (red→colourless) would be ideal for indicating an end-point for laser welding using an argon ion laser. This particular photochromic has a quantum yield of colouration of 0.2, and a quantum yield of decolouration of 0.055 $^{[122]}$. The quantum yield of fading of Aberchrome 540 is a factor of ten higher than the desired rate of fading required for use as an end-point indicator. If this photochromic was used with a similar concentration to the dye used in the earlier calculation, and irradiated under identical conditions, the compound would be converted to the ultraviolet absorbing form at a point where the heat generated at the tissue surface was below the protein denaturation temperature. The photochromic is too efficient. However, photochromic compounds with high quantum yields of decolouration are not automatically ruled out as suitable end point indicators. The concentration of Aberchrome could be increased. Although the number of photons absorbed by each molecule before photoisomerisation is significantly lower than the required value, the
fact that a higher concentration of photochromic is used, means that the number of molecules is higher than the ideal. Consequently, the same number of photons is used before all the molecules have been converted to the non absorbing form. The concentration of Aberchrome 540 required to show an end-point in the required temperature range would be.

No of molecules = \( \Phi_{\text{decolouration}} \times \text{No of photons} \)

No of molecules = 0.055 \times 2.218 \times 10^{16}

No of molecules = 1.22 \times 10^{17}

\[ \therefore \text{Concentration of Aberchrome 540 required = 0.5mol/dm}^3 \]

Although this concentration of Aberchrome may be difficult to obtain, the calculation shows that compounds with high quantum yields of decolouration can be considered as possible end-point indicators.

6.1.2. Thermochromics.

Thermochromic compounds are another class of compounds with potential for use as possible end-point indicators. Thermochromic materials undergo thermally induced transformations which are thermally reversible, producing a spectral change, typically (but not necessarily) of visible colour. Like photochromics, there are two possible ways in which thermochromic materials could be used to obtain a suitable end-point.

**Thermochromic indicators**—For this particular application, the thermochromic compound is designed to change colour at the blood vessel protein denaturation temperature. The colour change as a result of the temperature rise is used to indicate if the transition temperature has been reached on the laser irradiated tissue.

**COLOURLESS \( \leftrightarrow T<70^\circ\text{C} \) BLUE**

When the blood vessel protein denaturation temperature has been reached, a blue colour develops on the surface of the irradiated vessel.

**Thermochromic switches**—Alternatively, thermochromic compounds can be used as switching devices. Under these circumstances, if the transition occurs at the correct
temperature, it is impossible to over-irradiate the blood vessel tissue. At temperatures below the tissue denaturation temperature, the thermochromic should possess absorption characteristics resulting in absorption of the laser wavelengths, causing a corresponding temperature rise. However, once the transition temperature is reached, the thermochromic changes colour, there is no overlap between the absorption spectrum of the compound and the laser emission wavelengths. No further heat is generated. Bare and Mellon\textsuperscript{[123]} demonstrated by varying the proportions of ethanol, methanol and water in aqueous solutions of cobalt chloride, it was possible to use such a system as a liquid thermometer. The thermochromic nature of cobalt chloride in such a water/solvent system is as a result of ligand substitution at different temperatures.

The following equation described the colour changes involved.

\[ 2\text{EtOH} + 2\text{Cl}^- + (\text{Co(H}_2\text{O})_6\text{)}^{2+} \leftrightarrow (\text{Co(EtOH)}_2\text{Cl}_2) + 6\text{H}_2\text{O} \]  \(6.1\)

\[ \text{Pink} \leftrightarrow \text{Violet} \leftrightarrow \text{Blue} \]

\[ \text{Decreasing temperature} \quad \text{Increasing temperature} \]

A number of experiments were performed to assess the suitability of such a system. Although the colour changes would be applicable for the indication of an end-point, the presence of solutions of methanol and ethanol would be unsuitable for \textit{in vivo} vascular surgery.

6.1.3. \textbf{Photoreduction of dyes.}

The photoreducible property of certain dyes makes them suitable for use in improving the quality of anastomosis, by removing the uncertainty surrounding the determining of the end-point for irradiation. The simple scheme below outlines a typical photoreduction process.

\[ ^3\text{D}^* + \text{Re} \rightarrow \text{D}^{*\text{red}} + \text{Re}^{+\text{ox}} \rightarrow \text{Products} \]  \(6.2\)
where \( ^3D^* \) is the triplet state of the dye, Re the reducing agent, \( D^-_{\text{red}} \) the half reduced dye, \( Re^{+\text{ox}} \) the half oxidised reducing agent. A bimolecular photoreduction such as this has the advantage that the quantum yield of bleaching of the dye can be controlled by varying the concentration of the reducing agent.

The principle aims of the work described in this chapter were to investigate the possible photoreduction of two dyes Basic fuchsin, a triphenylmethane dye, and Eosin Y, a xanthene dye, (both previously established as suitable heat generators) using the reducing agents ascorbic acid and EDTA. With the aim of manipulating the systems, so that the quantum yield of fade of the dye was of the order \( 10^{-3} \), enabling such a system to be used to alleviate problems with over irradiation during laser welding. Steady state and time resolved techniques were utilised to determine values for the quantum yield of fade of various dye/reducing agent mixtures, and to investigate the mechanisms of photofading. Additionally, \textit{in vitro} end-to-end anastomosis of porcine splenic arteries were prepared using various mixtures of laser activated glues containing suitable reducing agents. The quality of vessel repair was assessed by bursting pressure studies, and the degree of thermal damage was assessed histologically. The principle objective of these \textit{in vitro} studies was to confirm that the photofading of chromophores, readily observed in solution could be suitably utilised for use in highly viscous solutions of bovine albumin applied to the surface of blood vessels.

6.2. \textbf{Experimental.}

6.2.1. The photofading of Basic fuchsin. Quantum yield of fade determination.

The fading experiments involving Basic fuchsin with the reducing agent ascorbic acid, were carried out with the dyes contained within solutions of human serum albumin. These experiments were performed in cuvettes rather than on the surface of biological tissue. Irradiation of the solutions was carried out using the Argon ion laser (Spectra Physics Model 2010, Hemel Hemstead, UK, 488/514nm), previously used for the preparation of ‘laser welds’. To ensure that the whole of the solutions were
irradiated, the beam of the laser was expanded using two additional lenses. This macroscopic measurement provides a simple model for assessing the fading rate of the same dyes when applied to the surface of a blood vessel.

The quantum yield of dye fade can be calculated from the dye loss as a result of a particular level of irradiation for a given time. The calculation of the quantum yield of fade is as follows.

If the absorbance of the dye solution at wavelength $\lambda$, is $A_1$ before irradiation, and the absorbance at the same wavelength following irradiation for $t$ seconds, is $A_2$, then it is possible to determine the number of molecules fading, if the molar extinction coefficient $\varepsilon$ of the dye at $\lambda$ is known, and a known volume $V$ of the dye has been irradiated.

$$\text{No of molecules fading} = \frac{A_1 - A_2 \times V}{\varepsilon} \times N_A$$

(6.3)

where $N_A$ is the Avogadro constant, $6.023 \times 10^{23}$ mol$^{-1}$, $V$ is in units of cm$^3$, and $\varepsilon$ has units of dm$^3$ cm$^{-1}$ mol$^{-1}$.

In order to determine the number of incident photons per second available for absorbance by the dye solution, the standard Potassium ferrioxalate actinometry system $[124,125]$ was used. The photochemical reaction of a ferrioxalate solution consists of the reduction of ferric ions to ferrous ions.

$$2\text{Fe}^{3+} + \text{C}_2\text{O}_4^{2-} \rightarrow 2\text{Fe}^{2+} + 2\text{CO}_2$$

(6.4)

A volume of potassium ferrioxalate solution ($V_1$) was placed in two cuvettes. One of which was irradiated for a time $t$, and the other was stored in the dark, but at the same temperature. Two volumetric flasks of total capacity $V_3$ were prepared consisting of a mixture of $2$ cm$^3$ 1,10-phenanthroline (0.1% solution) and $0.5$ cm$^3$ of a buffer solution (prepared from $600$ cm$^3$ of $1.0$ mol/dm Sodium acetate and $400$ cm$^3$ of $1$ M acetic acid). Following irradiation, two aliquots of $V_2$ were taken from the two separate cuvettes and added to the two volumetric flasks, at which stage, complexation of ferrous ions resulted. The volumetric flasks were stored in the dark for one hour, and the absorbance of the two samples was measured at $510$ nm. The
change in the absorbance was obtained by taking the difference, with the 'non irradiated' sample used as the reference.

The number of Fe$^{2+}$ ions formed during irradiation is given by

$$\text{No of Fe}^{2+} = \frac{N_A V_1 V_3 A_{510}}{10^3 V_2 I e_{510}}$$  \hspace{1cm} (6.5)

where $I$ is the optical pathlength of the cuvette used =1cm, $A_{510}$ is the change in absorbance at 510nm, $e_{510}$ is the molar extinction coefficient of the ferrous complex at 510nm (dm$^3$ cm$^{-1}$ mol$^{-1}$) and $V_1$, $V_2$ and $V_3$ in units of cm$^3$ are defined above.

Consequently, the number of photons per second can be calculated by

$$\text{No of photons per second} = \frac{\text{No of Fe}^{2+} \text{ formed}}{\phi_{\lambda}}$$  \hspace{1cm} (6.6)

where $\phi_{\lambda}$ is the quantum yield for production of the ferrous ions from Potassium ferrioxalate$^{[124]}$ and $t$ is the time of irradiation.

If the fraction of photons absorbed by the dye solution is defined as, $F = 1 - 10^{-A_s}$, so long as there is no wavelength dependency on the rate of fading, ie each photon absorbed results in fade with the same efficiency, it is possible to calculate the quantum yield of fade by,

$$\phi_{\text{FADE}} = \frac{\text{No of molecules fading}}{\text{No of Fe}^{2+} \text{ formed} \times F \times t}$$  \hspace{1cm} (6.7)

The irradiation power, and duration were adjusted to ensure that the extent of dye loss, and any possible production of photoproducts was kept to a minimum. Typically the change in absorption as a result of irradiation was of the order of 10%, reducing the error in the quantum yield of fade calculations.

For this set of experiments, the quantum yield of fade of Basic fuchsin was studied as a function of ascorbic acid concentration. For each irradiation, volumes of 3cm$^3$ within 1cm×1cm cuvettes were irradiated at a power of 1 watt for 1 minute.
6.2.1.1. **Results.**

The results from the fading experiments for basic fuchsin are shown in table 6.1. A stock solution of basic fuchsin in human serum albumin was used with a concentration of \(4.8 \times 10^{-4}\) mol/dm\(^3\), and a stock solution of ascorbic acid was used with a concentration of 1.1 mol/dm\(^3\).

<table>
<thead>
<tr>
<th>[Ascorbic acid] mol/dm(^3)</th>
<th>Mean number of photons per second</th>
<th>Irradiation duration seconds</th>
<th>Mean (\phi_{FADE})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(2.0 \times 10^{16})</td>
<td>60</td>
<td>0.0018</td>
</tr>
<tr>
<td>0.33</td>
<td></td>
<td>60</td>
<td>0.0038</td>
</tr>
<tr>
<td>0.55</td>
<td></td>
<td>60</td>
<td>0.0060</td>
</tr>
<tr>
<td>0.78</td>
<td></td>
<td>60</td>
<td>0.0093</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>60</td>
<td>0.0051</td>
</tr>
<tr>
<td>1.04</td>
<td>(3.2 \times 10^{16})</td>
<td>60</td>
<td>0.0027</td>
</tr>
<tr>
<td>1.26</td>
<td></td>
<td>60</td>
<td>0.0023</td>
</tr>
<tr>
<td>1.49</td>
<td></td>
<td>60</td>
<td>0.0021</td>
</tr>
<tr>
<td>1.72</td>
<td></td>
<td>60</td>
<td>0.0017</td>
</tr>
</tbody>
</table>

Table 6.1. Quantum yields of fade calculated for basic fuchsin in human albumin solution (4.8\(\times 10^{-4}\) mol/dm\(^3\)) with ascorbic acid.

It is important to note in these experiments, that the number of molecules fading is calculated from the change in absorbance at 488nm. It is assumed that because the conversion of the dye has been kept to a minimum \(\approx 10\%\), the rate of fade is constant during fading experiments. The calculation shown below details how the quantum yield of fade of basic fuchsin with 1.0 mol/dm\(^3\) ascorbic acid was determined.

Absorbance of dye solution before irradiation = 0.526
Absorbance of dye solution after irradiation = 0.474
Delta absorbance\(_{488nm}\) of dye solution = 0.052
Decadic absorption coefficient\(_{488nm}\) of dye = 2.42\(\times 10^4\) dm\(^3\)cm\(^{-1}\)mol\(^{-1}\)
No of molecules fading = 3.88\(\times 10^{15}\)
No of photons/second = \(3.2 \times 10^{16}\) (determined by actinometry)

Irradiation duration = 40 seconds

Fraction absorbed = \(1-10^{-A}\), = 0.702

Quantum yield of fade = 0.0043

---

Figure 6.2: Plot of the quantum yield of fade of Basic fuchs\(\text{in}\) (4.8\(\times 10^{-4}\)mol/dm\(^3\)) as a function of Ascorbic acid concentration.
6.2.1.2. **Discussion.**

The principle objective of this experiment was to determine the quantum yield of fading of human albumin solutions of Basic fuchsin as a function of ascorbic acid concentration, with a view to using such a system for the determination of a suitable end-point for laser welding. The results illustrated in figure 6.2, reveal that the quantum yield of fade of Basic fuchsin is of the order of $10^{-3}$. These values are in close agreement with the predicted required quantum yield of fade for a dye to be used to generate the desired end-point for laser welding. As the Ascorbic acid concentration increased to 0.78 mol/dm³ the quantum yield of fade increased from 0.0018 to 0.0093. However, further increase beyond an ascorbic acid concentration of 0.78 mol/dm³ resulted in a sudden fall in the yield of fade, from 0.0093, to 0.0017 (1.72 mol/dm³).

During nanosecond flash photolysis studies, the absorption due to the triplet state of Basic fuchsin was not observed. In a review article by Duxbury [126] where the photophysical properties of triphenylmethane dyes are discussed, it is suggested that rapid internal conversion in low viscosity solutions caused the intersystem crossing process to the triplet state to be low. There are several mechanisms in which dye fading can be explained [127]. Photoreduction competes with photooxidation to provide the most favoured path for degradation of triphenylmethane dyes. In general, dye photoreduction is more favoured in anaerobic conditions, and in the presence of compounds that are oxidised more readily than the dye. It has been suggested [128] that the fading of triphenylmethane dyes within proteins is associated with a photoreduction mechanism. A number of studies have suggested that the excited triplet state of the dye is responsible for the photoreduction of triphenylmethane dyes [129,130,131,132]. However, although this explanation may be reasonable for fading of chromophores in cases where the rotation of phenyl rings is impeded, Picosecond studies [133] on Crystal violet, Parafuchsin and Ethylviolet revealed that less than 3% of the molecules populated the triplet state. To further confirm the involvement of the excited triplet state in the photoreduction of triphenylmethane dyes, Allen [132] observed that the presence of a triplet sensitiser, benzophenone, increased the rate of
photoreduction of Malachite green in polyvinyl alcohol. A number of other dyes were studied and it was suggested that the mechanism for photofading, was that following absorption of light, the excited singlet state underwent intersystem crossing to the excited triplet state, which ejected an electron. This ejected electron reduced the ground state dye to form a triphenylmethyl radical. Under anaerobic conditions, it was found that the production of triphenylmethyl radicals as a result of dye photoreduction in solutions of 2-propanol, could be inhibited by the introduction of oxygen. \(^{132}\).

Several workers have found a strong correlation between the phosphorescence and photoreduction of dyes \(^{129,130,134}\). In particular symmetric triphenylmethane dyes were prone to photoreduction by white light. As mentioned above, an increase in the molecular rigidity of a dye encourages the population of the excited triplet state. Following on from these observations Oster \(^{129,130}\) observed that the dyes phosphorescence and photoreduction increased when the dyes were present in highly viscous media such as polymethacylic acid. In the unbound state a number of triphenylmethane dyes including Acidic fuchsin could not be photoreduced in the presence of the reducing agent ascorbic acid. However, when polymethacylic acid was added to the system, the dyes were photoreduced irreversibly giving a colourless solution with a new absorption maximum in the ultraviolet region. In addition, the phosphorescence of several triphenylmethane dyes studies was quenched by ascorbic acid, although fluorescence remained unchanged. Flash photolysis studies confirmed these findings by revealing that a metastable state (most likely the triplet state) was detected only when there was a high concentration of polymer.

As a result of these studies \(^{130}\), the following reaction scheme for the photofading of triphenylmethane dyes (including Basic fuchsin) in the bound state in the presence of a reducing agent R was presented.

\[
D \rightarrow \text{D}^* \quad (6.8)
\]

\[
\text{D}^* \rightarrow \text{D} + \text{heat and or fluorescence} \quad (6.9)
\]

\[
\text{D}^* \rightarrow \text{3D}^* \quad (6.10)
\]

\[
\text{D}^* + \text{D} \rightarrow \text{3D}^* + \text{D} \quad (6.11)
\]
The photochemical fading of dyes - A possible end-point for laser assisted vascular anastomosis.

\[ ^3D^* \rightarrow D + \text{phosphorescence} \]  
(6.12) 
\[ ^3D^* + R \rightarrow D + R \]  
(6.13) 
\[ ^3D^* + R \rightarrow [\text{complex}] \]  
(6.14) 
\[ [\text{complex}] \rightarrow \text{colourless product} \]  
(6.15)

Furthermore, studies have revealed that the rate of dye fading may be affected by temperature\[^{129}\]. For mixtures of Acidic fuchsin within glucose glass, the rate of dye photoreduction increased up to a temperature of 60°C, but then decreased as the temperature was raised beyond this point. To explain this observation, three factors were considered that were in keeping with kinetic and spectral results.

- There are a number of molecules that are converted to the metastable triplet state.
  
  The greater the viscosity of the glass (hence the lower the temperature) there is less internal conversion from the singlet state to the ground state. Consequently there are a greater number of molecules that undergo transition to the triplet state.

- The lifetime of the metastable state is increased when the temperature is lowered, (the fluorescence intensity decreases with increasing temperature).

- The two effects described above are opposed by the contribution of the activation energy barrier required to be overcome for the triplet state of the dye to react with the glucose glass.

The combination of these three factors leads to an optimum rate of fade. It is important to realise that the mechanism of dye fading in the solution phase may well be different to fading of dyes in the solid state.  

By studying the light fastness of triphenylmethane dyes on synthetic polymers and protein substrates, Duxbury\[^{133}\] found that dye fading was photosensitised by components of the protein structure. The specific groups of interest were the guanido group of arginine and the carboxylic groups of glutamic and aspartic acid. However, lysine, histidine or the excited singlet state of oxygen did not appear to sensitize the fading of dyes within protein substrates. The sensitised photoreduction of dyes was believed to proceed by a one step transfer (of an electron and a hydrogen atom) and an exciplex mechanism. Electron spin resonance studies reinforced these beliefs for
the dye fading in the presence of acetate groups, but not for the fading in the presence of guanido groups.

In summary, in the previous fading experiments with triphenylmethane dyes, using steady state irradiation techniques, it has been shown that dye fading occurs in aerobic and anaerobic conditions, although the rate of reaction with degassed solutions was greater. It has been suggested that photoreduction is enhanced when the dye is in a bound form, i.e., to a polymer or protein. As a result of various phosphorescence, fluorescence and flash photolysis experiments, it has been suggested that the excited triplet state of the dye is the species responsible for photoreduction. However, it is believed that the triplet excited state does not directly react with the reductant, rather that a complex is formed as a result of reaction between triplet and reductant. This complex reacts further to give the colourless product.

The results of this investigation have proved how air saturated solutions of Basic fuchsin in a protein substrate, human serum albumin, together with the reducing agent ascorbic acid, can be faded by irradiation with an argon laser. The quantum yield of fade of Basic fuchsin was found to be of a similar order of magnitude to that required to give a suitable end point for laser vascular anastomosis. Therefore, the use of such a system would be useful to alleviate the risk associated with the over irradiation of blood vessels.

6.2.2. The photofading of Eosin Y. Quantum yield of fade determination.

The previous section demonstrated how ascorbic acid could be used as the reducing agent for the photofading of Basic fuchsin. In an attempt not to limit the potential end point indicators to one specific dye, which may be unsuitable for the use in the in vivo repair of blood vessels, a further system with potential in the area of laser radiation control has been investigated.

The fading experiments involved Eosin Y, a xanthene dye, with the reducing agent EDTA. The measurements of the quantum yield of fade were performed on dye solutions buffered to a pH of 5. None of these experiments were performed using biological tissue.
Irradiation of each solution was carried out using a 300 watt ozone free xenon arc lamp (Oriel 6258) with a 532nm interference filter interposed between the arc lamp and the sample to cut off the unwanted wavelengths. It was hoped that these measurements would provide a means of assessing the fading rate of Eosin Y when applied to the surface of a blood vessel. As described in a previous section, the quantum yield of dye fade can be calculated from the dye loss as a result of a particular level of irradiation for a given time. Calculations for determination of the quantum yield of fade are presented in section 6.2.2. The Potassium ferrioxalate system described earlier was used to determine the number of photons per second available for absorption by the dye solution.

For investigation of the photofading of Eosin Y, the quantum yield of fade was studied as a function of both EDTA and eosin Y concentrations. Because of variation in the dye concentration and rate of fading, a range of quartz cuvettes, dye volumes and irradiation durations were used.

6.2.2.1. Results.

The results from the fading experiments for Eosin Y with EDTA are shown in the proceeding tables 6.2-6.9. The results illustrate the variation in quantum yield of fade with increasing EDTA concentration.

<table>
<thead>
<tr>
<th>[EDTA]/mol/dm³</th>
<th>Mean number of photons per second</th>
<th>Irradiation duration/seconds</th>
<th>Mean $\Phi_{FADE}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1.56 \times 10^{14}$</td>
<td>300</td>
<td>0.0166</td>
</tr>
<tr>
<td>0.034</td>
<td></td>
<td>300</td>
<td>0.0158</td>
</tr>
<tr>
<td>0.068</td>
<td></td>
<td>300</td>
<td>0.022</td>
</tr>
<tr>
<td>0.102</td>
<td></td>
<td>300</td>
<td>0.066</td>
</tr>
<tr>
<td>0.136</td>
<td></td>
<td>300</td>
<td>0.104</td>
</tr>
<tr>
<td>0.17</td>
<td></td>
<td>300</td>
<td>0.158</td>
</tr>
</tbody>
</table>

Table 6.2. Variation in quantum yield of fade of Eosin Y ($6.0 \times 10^{-2}$mol/dm³) with EDTA concentration.
Chapter Six: The photochemical fading of dyes - A possible end-point for laser assisted vascular anastomosis.

<table>
<thead>
<tr>
<th>[EDTA]/mol/dm³</th>
<th>Mean number of photons per second</th>
<th>Irradiation duration/ seconds</th>
<th>Mean φ\textsubscript{FADE}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.49×10¹⁴</td>
<td>300</td>
<td>0.039</td>
</tr>
<tr>
<td>0.034</td>
<td></td>
<td>180</td>
<td>0.033</td>
</tr>
<tr>
<td>0.068</td>
<td></td>
<td>180</td>
<td>0.093</td>
</tr>
<tr>
<td>0.102</td>
<td></td>
<td>120</td>
<td>0.171</td>
</tr>
<tr>
<td>0.136</td>
<td></td>
<td>60</td>
<td>0.112</td>
</tr>
<tr>
<td>0.17</td>
<td></td>
<td>60</td>
<td>0.133</td>
</tr>
</tbody>
</table>

Table 6.3. Variation in quantum yield of fade of Eosin Y (1.42×10⁻⁴mol/dm³) with EDTA concentration.

<table>
<thead>
<tr>
<th>[EDTA]/mol/dm³</th>
<th>Mean number of photons per second</th>
<th>Irradiation duration/ seconds</th>
<th>Mean φ\textsubscript{FADE}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.1×10¹⁴</td>
<td>300</td>
<td>0.046</td>
</tr>
<tr>
<td>0.034</td>
<td></td>
<td>300</td>
<td>0.036</td>
</tr>
<tr>
<td>0.068</td>
<td></td>
<td>300</td>
<td>0.098</td>
</tr>
<tr>
<td>0.102</td>
<td></td>
<td>300</td>
<td>0.15</td>
</tr>
<tr>
<td>0.134</td>
<td></td>
<td>150</td>
<td>0.25</td>
</tr>
<tr>
<td>0.17</td>
<td></td>
<td>150</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Table 6.4. Variation in quantum yield of fade of Eosin Y (2.7×10⁻⁴mol/dm³) with EDTA concentration.

<table>
<thead>
<tr>
<th>[EDTA]/mol/dm³</th>
<th>Mean number of photons per second</th>
<th>Irradiation duration/ seconds</th>
<th>Mean φ\textsubscript{FADE}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.3×10¹³</td>
<td>300</td>
<td>0.042</td>
</tr>
<tr>
<td>0.034</td>
<td></td>
<td>300</td>
<td>0.0525</td>
</tr>
<tr>
<td>0.068</td>
<td></td>
<td>300</td>
<td>0.081</td>
</tr>
<tr>
<td>0.102</td>
<td></td>
<td>180</td>
<td>0.155</td>
</tr>
<tr>
<td>0.136</td>
<td></td>
<td>120</td>
<td>0.225</td>
</tr>
<tr>
<td>0.17</td>
<td></td>
<td>120</td>
<td>0.370</td>
</tr>
</tbody>
</table>

Table 6.5. Variation in quantum yield of fade of Eosin Y (3.45×10⁻⁴mol/dm³) with EDTA concentration.
Chapter Six: The photochemical fading of dyes - A possible end-point for laser assisted vascular anastomosis.

<table>
<thead>
<tr>
<th>[EDTA]/mol/dm$^3$</th>
<th>Mean number of photons per second</th>
<th>Irradiation duration/seconds</th>
<th>Mean $\phi_{\text{FADE}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1.3 \times 10^{14}$</td>
<td>300</td>
<td>0.040</td>
</tr>
<tr>
<td>0.034</td>
<td></td>
<td></td>
<td>0.056</td>
</tr>
<tr>
<td>0.068</td>
<td></td>
<td></td>
<td>0.066</td>
</tr>
<tr>
<td>0.102</td>
<td></td>
<td></td>
<td>0.154</td>
</tr>
<tr>
<td>0.136</td>
<td></td>
<td></td>
<td>0.207</td>
</tr>
<tr>
<td>0.17</td>
<td></td>
<td></td>
<td>0.335</td>
</tr>
</tbody>
</table>

Table 6.6 Variation in quantum yield of fade of Eosin Y ($4.7 \times 10^4$ mol/dm$^3$) with EDTA concentration.

<table>
<thead>
<tr>
<th>[EDTA]/mol/dm$^3$</th>
<th>Mean number of photons per second</th>
<th>Irradiation duration/seconds</th>
<th>Mean $\phi_{\text{FADE}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1.3 \times 10^{14}$</td>
<td>300</td>
<td>0.032</td>
</tr>
<tr>
<td>0.034</td>
<td></td>
<td></td>
<td>0.040</td>
</tr>
<tr>
<td>0.068</td>
<td></td>
<td></td>
<td>0.045</td>
</tr>
<tr>
<td>0.102</td>
<td></td>
<td></td>
<td>0.096</td>
</tr>
<tr>
<td>0.136</td>
<td></td>
<td>180</td>
<td>0.085</td>
</tr>
<tr>
<td>0.17</td>
<td></td>
<td>180</td>
<td>0.204</td>
</tr>
</tbody>
</table>

Table 6.7. Variation in quantum yield of fade of Eosin Y ($5.9 \times 10^4$ mol/dm$^3$) with EDTA concentration.

<table>
<thead>
<tr>
<th>[EDTA]/mol/dm$^3$</th>
<th>Mean number of photons per second</th>
<th>Irradiation duration/seconds</th>
<th>Mean $\phi_{\text{FADE}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1.6 \times 10^{14}$</td>
<td>300</td>
<td>0.022</td>
</tr>
<tr>
<td>0.034</td>
<td></td>
<td></td>
<td>0.018</td>
</tr>
<tr>
<td>0.068</td>
<td></td>
<td></td>
<td>0.017</td>
</tr>
<tr>
<td>0.102</td>
<td></td>
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</tr>
<tr>
<td>0.136</td>
<td></td>
<td></td>
<td>0.066</td>
</tr>
<tr>
<td>0.17</td>
<td></td>
<td>240</td>
<td>0.096</td>
</tr>
</tbody>
</table>

Table 6.8. Variation in quantum yield of fade of Eosin Y ($7.45 \times 10^4$ mol/dm$^3$) with EDTA concentration.
Table 6.9. Variation in quantum yield of fade of Eosin Y (9.8x10^4 mol/dm$^3$) with EDTA concentration.

The plot shown in figure 6.3 shows the variation on the quantum yield of fade of Eosin Y with EDTA concentration. It is important to note in these experiments, that the number of molecules fading is calculated from the change in absorbance at 532nm, the irradiating wavelength. It is assumed that because the conversion of dye has been kept to a minimum, =10%, the rate of fade is constant during the fading experiments.
Chapter Six: The photochemical fading of dyes. A possible end-point for laser assisted vascular anastomosis.

Figure 6.3: Plot of the variation in the quantum yield of fade of eosin as a function of dye and reducing agent concentration.
6.2.2.2. **Discussion.**

It is readily observable from the results illustrated in figure 6.3, that as the EDTA concentration increases, there is a corresponding increase in the quantum yield of fade. Additionally, it is noticeable that the rate of fading of dye increases with increasing dye concentration up to a certain point. As the dye concentration increases beyond this point, there is a decrease in the quantum yield of fade. The most likely explanation to the observed trend is that there is self quenching of Eosin Y excited triplet state with the ground state (see section 6.2.3).

In addition to steady state irradiation experiments, time resolved measurements were performed using flash photolytic techniques. Such measurements were undertaken with a view to explaining the observed trends in the quantum yield of fade of solutions of Eosin Y.

6.2.3. **Nanosecond flash photolysis experiments.**

The methods of data collection using the JK2000 Q-switched Nd:YAG laser system have previously been described in section 1.7.2. Flash photolytic methods were used to illustrate that self quenching was occurring between the excited triplet state of Eosin Y and the ground state of the dye. Experiments were performed with solutions contained in 1cm×1cm quartz cuvettes. In certain instances, solutions were degassed. In these cases 1mm pathlength cuvettes were cycled through three successive freeze pump thaw procedures to a pressure of approximately 1×10⁻³ mbar. These procedures were performed on a vacuum line. The concentration of dye used for these experiments ranged from 8×10⁻⁵ mol/dm³ to 4×10⁻⁴ mol/dm³. Flash excitation of each solution at 532nm (the second harmonic of a Nd:YAG laser) resulted in the triplet state of Eosin Y decaying by a first order process. The kinetics of the triplet state decay, together with the ground state depletion were monitored at 610 and 510nm respectively.
Chapter Six: The photochemical fading of dyes- A possible end-point for laser assisted vascular anastomosis.

6.2.3.1. Results.

The results of these self quenching measurements are shown in figure 6.4. The plot of dye concentration against first order rate constant, yielded a straight line with a gradient of $1.24 \times 10^9 \text{dm}^3\text{mol}^{-1}\text{s}^{-1}$. The results confirm that as the Eosin Y concentration increases, the lifetime of the excited triplet state decreases. Consequently, the chance of an encounter between the triplet state of Eosin Y and EDTA decreases. To a certain extent this explains the observed decrease in quantum yield of fade with increasing dye concentration. Furthermore, a number of experiments were performed using the flash photolysis apparatus to investigate the kinetics of the reaction between Eosin Y and EDTA. Degassed solutions containing a fixed Eosin Y concentration ($1.6 \times 10^{-6}\text{mol/dm}^3$), but increasing EDTA concentrations were flash excited. The kinetics of the decay of the triplet excited state $\lambda=570\text{nm}$ were monitored. Studies revealed that an increase in reducing agent concentration did not affect the kinetics of the triplet state decay. There was no evidence to suggest that quenching of the triplet state was taking place (see figure 6.5). However interestingly, it was noticeable that as the EDTA concentration increased, the initial excited state absorbance was reduced. These results suggested the possibility of the excited singlet state being quenched by EDTA. Consequently, the fluorescence intensities of degassed solutions containing a fixed Eosin Y concentration ($1 \times 10^{-5}\text{mol/dm}^3$), with increasing EDTA concentration ($6 \times 10^{-2} \rightarrow 2 \times 10^{-1}\text{mol/dm}^3$) were measured using a fluorimeter. However, the results of fluorescence measurements suggested that quenching of the excited singlet state of Eosin Y was not occurring, as the fluorescence intensity was unaffected by increasing EDTA concentration, see figure 6.6.
Figure 6.4: Plot of the triplet state deactivation rate constant of Eosin Y (probed 570nm) as a function of dye concentration.
Figure 6.5: Transient difference data traces for Eosin Y in aqueous solution, for three EDTA concentration, probed at 570nm.
Figure 6.6: Fluorescence emission spectra of Eosin Y (excitation wavelength 455nm) studied as a function of EDTA concentration (6x10^{-2}-2x10^{-1}mol/dm^3).
6.2.3.2. Discussion.

The primary aim of this set of experiments was to determine the quantum yield of fade of buffered solutions of Eosin Y as a function of reducing agent and dye concentration. It was hoped that this system could be used as a suitable end-point indicator for ‘laser welding’. The results indicate that as the EDTA concentration increases, there is a corresponding increase in the quantum yield of fade. However, interestingly, the relationship between the rate of fade and Eosin Y concentration was not straightforward. The results of numerous studies revealed that, in general the quantum yield of fade increased to a particular value, but a further increase in dye concentration resulted in a reduction in the quantum yield of fade. The quantum yield of fade of Eosin Y solutions ranged from 0.016 to 0.37, a diverse range. It would be acceptable to assume that the concentration of dye and reducing agent could be altered, resulting in a system with the desired yield of fading \(10^3\), for use in the determination of a suitable end-point for laser welding.

As a result of flash photolysis experiments on dilute solutions of Eosin y and EDTA, the following mechanism has been proposed to explain the reason why at a fixed dye concentration, with increasing EDTA concentration the following is observed.

- The fluorescence yield of Eosin Y is unaffected.
- The kinetics of Eosin Y triplet decay are unaffected.
- The amount of triplet Eosin is reduced.

Since high dye concentrations were used during steady state quantum yield determinations and flash photolysis studies, it is likely that dimerisation of Eosin Y is occurring \([136]\). Therefore the photobleaching of Eosin Y by EDTA may be explained through the Eosin Y dimer pathway.
It is evident from the proposed mechanism that because the singlet state Eosin Y monomer is populated directly by excitation of the monomer ground state it is unaffected by the dimer route and the addition of EDTA. The triplet state of the Eosin Y monomer is populated through two different pathways, by intersystem crossing from the singlet monomer, or by dissociation of the triplet state dimer. As the EDTA concentration is increased, the reaction between $^3D^*$ and EDTA competes with the dissociation pathway, consequently the population of $^3M^*$ by dissociation is decreased. This mechanism can also be used to explain the reason why the quantum yield of fade of Eosin Y decreases as the ground state Eosin Y concentration increases (see figure 6.3). As the dye concentration is increased, the self quenching reaction ($^3D^* + M$) begins to compete with the Eosin Y/EDTA pathway ($^3D^* + \text{EDTA} \rightarrow \text{Products}$). Consequently, the quantum yield of fade is reduced.

Further flash photolysis measurements were required to prove this mechanism, however the principle objective of this particular study was to determine the quantum yield of fade of Eosin Y, for application with laser tissue welding. Due to time constraints, it was felt more important to attempt further laser welding experiments utilising laser activated glues consisting of specific combinations of Eosin Y and EDTA.
6.3. **Bursting pressure study III. Assessment of the Eosin Y/EDTA system to be used as a possible end point indicator for laser assisted vascular anastomosis.**

6.3.1. **Introduction.**

In the preceding sections of this chapter, it was shown how solutions of Basic fuchsin and eosin Y (two chromophores already established as suitable temperature generating dyes) could be faded with a quantum yield of fade comparable to that necessary for use in the laser welding of blood vessels. Although these studies were useful in establishing that Basic fuchsin and Eosin Y could be photofaded at the desired rate, they were studied on a macroscopic scale with the solutions confined to cuvettes rather than on the surface of blood vessels. For this reason, the ability of the Eosin Y/EDTA system to be used as a possible end-point indicator was assessed by preparing end-to-end anastomoses in porcine splenic arteries using laser activated glues with/without the reductant EDTA. Mechanical bursting pressure tests were used to measure the strength of repaired vessels. A number of anastomosis were retained for histological examination.

Prior to the fashioning of anastomoses, a number of solutions of Eosin Y/EDTA in bovine serum albumin were prepared, with concentrations in accordance with previous fading experiments. These viscous mixtures were irradiated under similar conditions to those used for the preparation of laser welds, ie 0.2 watts, 5 second/spot irradiation duration, 1mm spot size. The concentration of dye and reducing agent were varied so the fading of Eosin Y was complete before thermal damage of the blood vessel protein ensued.

6.3.2. **Materials and methods.**

Porcine splenic arteries were collected and prepared as described in section 4.2. End-to-end anastomoses were carried out on short lengths of artery dissected transversely. The separated vessel was supported on a fine stainless steel high pressure gas tube.
The vessel edges were drawn together using two horizontal mattress sutures (Surgibond™ 5/0). Final preparation of the anastomoses and irradiation continued as described in earlier sections. A range of laser powers (0.15-0.3 watts) were used to give the optimum bursting strength, while minimising the extent of thermal damage, particularly for the non-fading laser activated glues.

6.3.2.1. Bovine albumin.
As a result of previous investigations into the dependence of bovine albumin concentration on the strength of longitudinal arteriotomies repaired by laser, (section 4.3.2), a bovine albumin concentration of 40%w/w was used throughout these studies. Preparation of this solution has been described in an earlier chapter.

6.3.2.2. Chromophore and reducing agent.
Only Eosin Y was used in combination with bovine albumin to create a laser activated glue. Additionally, in certain instances, EDTA (Aldrich Chemical Co) was added to mixtures of dye and bovine albumin to generate a laser activated glue with the ability to be photofaded. The concentrations of Eosin Y and EDTA within a typical laser activated glue were in the region of $2 \times 10^{-2}$ mol/dm$^3$ and 0.08 mol/dm$^3$ respectively.

6.3.2.3. Measurement of bursting pressure.
Immediately following repair, each anastomosis was subjected to a series of bursting pressure tests. The bursting pressure apparatus and procedures have been described in an earlier chapter. (See section 4.2.3 page 124).

6.3.3. Results.
Before attempting to prepare end-to-end anastomosis for bursting pressure assessment, a number of experiments were performed to establish the concentrations of Eosin Y and EDTA required to give a suitable end point. Studies were limited to applying mixtures of dye/albumin and EDTA to the surface of microscope slides and irradiating using the argon laser system. Attempts were made to achieve a constant
thickness of laser glue on the surface of the microscope slides, comparable to that used on the surface of blood vessels.

As a result of a number of experiments using a range of Eosin/EDTA concentrations, the optimum combination of Eosin Y and EDTA concentration was \(2 \times 10^{-2}\) mol/dm\(^3\) and 0.083 mol/dm\(^3\) respectively. Laser glue solutions without the addition of EDTA irradiated at a laser power of 0.2 watts showed visible signs of coagulation after a period of 5 seconds. Irradiation beyond this point resulted in definite carbonisation of the bovine albumin mixture. Conversely solutions of laser activated glue with EDTA irradiated under identical conditions, 0.2 watts, faded in a time scale comparable to the coagulation time. Furthermore, because of fading of the glue, this particular solution could not be over irradiated. Irradiation durations approaching 20 seconds were used, with no evidence to suggest any albumin charring.

The results of the bursting pressure studies using a range of laser powers and two different laser activated glues are presented in the proceeding sections. Table 6.10 shows the results of the mechanical tests of the end-to-end anastomosis repaired at four different power setting with Eosin Y laser activated glue without reducing agent.

<table>
<thead>
<tr>
<th>P/ watts</th>
<th>Power density/ watts/cm(^2)</th>
<th>Mean irradiation duration to end point/ seconds</th>
<th>Mean bursting pressure/ mmHg (n=10)</th>
<th>Range of bursting pressures/mm Hg</th>
<th>Confidence limits (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>19</td>
<td>10</td>
<td>357.9</td>
<td>185-585</td>
<td>157-559</td>
</tr>
<tr>
<td>0.2</td>
<td>26</td>
<td>&lt;5</td>
<td>392.4</td>
<td>128-538</td>
<td>294-491</td>
</tr>
<tr>
<td>0.25</td>
<td>32</td>
<td>&lt;3</td>
<td>380.2</td>
<td>130-539</td>
<td>284-477</td>
</tr>
<tr>
<td>0.3</td>
<td>38</td>
<td>1</td>
<td>317.8</td>
<td>206-469</td>
<td>160-476</td>
</tr>
</tbody>
</table>

Table 6.10. Table showing the burst pressures obtained for the anastomoses repaired using an Eosin Y/bovine albumin laser activated glue, without EDTA.

For these particular laser welds, a visual end-point was used as an indication for the termination of irradiation. Irradiation was continued until a glowing of the tissue surface was observed. The optimum power densities in this category of repair were 26 and 32W/cm\(^2\) with average bursting pressures of 392 and 380 mmHg respectively.
All mean bursting pressures were consistently above the normal physiological range (>300mmHg). In a number of cases, particularly using a power of 0.2 watts, the anastomosis did not burst at all, in these instances bursting pressure testing was stopped because of leaks from tiny side branch holes in the length of artery. Conversely, when repairs were prepared using a laser power of 0.25 watts, and particularly 0.3 watts, the onset of vessel coagulation occurred almost instantaneously. Control of the tissue temperature was difficult, and vessel carbonisation was widespread, see figure 6.7 compared to figure 6.8. The occurrence of thermal damage reduced the strength of laser repairs. Invariably, anastomoses leaked from areas that were obviously over irradiated.

Table 6.11 shows the results of the mechanical tests of the end-to-end anastomosis repaired at four different power settings, with Eosin Y laser activated glue with EDTA.

<table>
<thead>
<tr>
<th>Power density/ watts/cm²</th>
<th>Mean bursting pressure/ mmHg (n=10)</th>
<th>Range of bursting pressures/ mmHg</th>
<th>Confidence limits (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 19</td>
<td>319.9</td>
<td>284-425</td>
<td>245-395</td>
</tr>
<tr>
<td>0.2 26</td>
<td>463.5</td>
<td>265-727</td>
<td>372-551</td>
</tr>
<tr>
<td>0.25 32</td>
<td>410.4</td>
<td>277-588</td>
<td>351-469</td>
</tr>
<tr>
<td>0.3 38</td>
<td>407.0</td>
<td>246-544</td>
<td>248-566</td>
</tr>
</tbody>
</table>

Table 6.11. Table showing the burst pressures obtained for the anastomoses repaired using Eosin Y/bovine albumin laser activated glue with EDTA.

The anastomoses repaired with a laser glue consisting of EDTA capable of photofading were not irradiated for a particular exposure time. It was unnecessary to use a visual end-point as an indication for the termination of irradiation. As a result of dye fading, it was impossible to over irradiate these particular anastomoses. The optimum power density in this category of repair was 26W/cm², with an average
bursting pressure of 464 mmHg. All mean bursting pressures were consistently above the normal physiological range (>300 mmHg). In fact, all repaired vessels were able to withstand pressures greater than or equal to 250 mmHg. Similar to the first group of welds, in the present bursting pressure studies, there were several anastomoses that did not burst. In these instances, bursting pressure testing stopped prematurely as a result of leaks from side branch holes. As a result of dye fading on the surface of the splenic artery, welds prepared using glues with EDTA showed little sign of thermal damage. Consequently the strength of welds was unaffected as a result of thermal damage. Figure 6.7, 6.9, 6.10 illustrate the difference in blood vessel appearance as a result of laser repair using laser activated glue with and without EDTA. Vessels repaired without EDTA showed definite signs of excessive thermal damage on the blood vessel surface around the anastomoses. However, the welds fashioned with EDTA showed very little, if any thermal damage. Additionally, it was noticeable that the laser glue around the anastomoses showed significant signs of fading when compared to glues prepared without EDTA.
Chapter Six: The photochemical fading of dyes - A possible end-point for laser assisted vascular anastomosis.

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Figure 6.7: Photograph of an artery repaired using an Eosin Y laser activated glue, without EDTA, irradiated at a power of 0.3W. Showing evidence of thermal damage.
Figure 6.8: Photograph of an artery repaired using an Eosin Y laser activated glue, without EDTA, irradiated at a power of 0.2W. No evidence of thermal damage.
Figure 6.10. Photograph showing the difference in blood vessel appearance for arteries repaired using an Eosin Y laser activated glue at a laser power of 0.25W, with (right hand), and without (left hand) reducing agent EDTA.
6.3.4. Discussion.

The primary objective of this set of experiments was to investigate if EDTA could be combined with a laser activated glue consisting of Eosin Y and bovine albumin, to reinforce anastomosis prepared by laser without resulting in over irradiation of the blood vessel proteins, by acting as an end-point switch. The repair of end-to-end anastomoses in porcine splenic arteries was assessed by bursting pressure. Two different irradiation conditions were studied. Forty anastomoses were prepared using 4 different laser powers with the addition of a laser activated glue without EDTA, and a further forty anastomoses were fashioned at the same laser powers using a laser activated glue with EDTA. There was no significant difference between the bursting pressure of the two laser glue groups. All mean bursting strengths were in excess of 300mmHg, however, the maximum burst pressure was slightly higher for the ‘with EDTA’ repairs (464mmHg) compared to the ‘without EDTA’ group (392mmHg).

The most significant difference between the two groups was the degree of thermal damage. Little thermal damage was noticeable when anastomoses were fashioned with EDTA, even when the highest laser powers of 0.25 and 0.3 watts were used.

In conclusion, it has been shown that a laser activated glue able to be photobleached can be applied to the surface of a blood vessel, providing consistent anastomoses at a range of laser powers. These vessel repairs were able to withstand typical intraluminal pressures, without the incidence of extensive thermal damage to the blood vessels as a consequence of inadvertent over-irradiation. These experiments are the first reported studies whereby a suitable end-point has been achieved using a laser activated glue to fashion vascular anastomoses.
7. A HISTOLOGICAL STUDY OF LASER ASSISTED VASCULAR ANASTOMOSIS USING A LASER ACTIVATED ADHESIVE.

7.1 Introduction

In the previous two chapters it has been shown how a laser activated glue could be used in conjunction with an Argon ion laser to effect end-to-end anastomoses that were able to withstand pressures well in excess of physiological systolic pressures. In each case the optimum results were generated by the Argon laser with chromophore/bovine albumin mixtures. In the present chapter, attempts were made to evaluate the effects of laser activated tissue glues on porcine tissue. Samples were prepared and were subject to histological examination.

All blood vessels have a similar basic structure (see section 1.1.2), however this skeleton may be modified by the function of the vessel. The most obvious difference is that between veins and arteries. Veins have thinner walls than arteries because they are not subjected to high pressures. Blood vessels have three layers, from the outer layer to the inner surface (the blood surface). The outer layer, the adventitia consists of a loose network of connective tissue and small blood vessels known as the vasa vasorum "the vessels of vessels" which communicate to the intimal surface. During microscopic examination it was observed that most of the adventitia had been dissected away, consequently it was not evident on any of the histological sections. Between the adventitia and media lies a layer of elastic tissue, the external elastic lamina. This layer was particularly thick for the vessels examined in this species. The media consists of smooth muscle cells arranged in a circular manner. These cells are arranged in small groups and are separated by collagen, for strength, and elastin for extensibility/contraction. The thickness of the media varies widely depending on species and function. It was observed that the porcine splenic artery had a very thick media. The media is separated from the innermost layer by a further elastic layer, the internal elastic lamina. For the arteries studied the internal elastic lamina was less dense than the external lamina. The internal elastic lamina is covered by the intima,
which consists of a single layer of endothelial cells. This layer is particularly fragile and was not identifiable in this study as it had been removed as a result of slaughtering and subsequent evisceration of the pigs.

7.2. Methods

After anastomoses were prepared and subsequently burst pressure tested, the tissues were fixed by immersion in 10% aqueous formalin in order to preserve the tissue and prepare the artery for further processing. By soaking the tissue with increasing concentrations of alcohol, the tissue water was gradually replaced. The samples were then soaked in xylene (miscible with paraffin wax), then impregnated with paraffin wax to allow cutting of the sections for microscopic examination. The sections, usually cut at 4 microns thickness were mounted on microscope slides prior to staining. Staining of the tissue facilitates identification of the main tissue elements as a result of a chemical combination. Throughout this study, the universally employed Haematoxylin and Eosin combination was used. This system colours the cell nuclei blue, and other structures shades of pink and orange/red. Additionally a special stain, Elastic Van Gieson was employed, this comprises of a combination of haematoxylin nuclear stain and Acidic fuchsin counterstain. This staining gives blue/black nuclei, black elastin, pink/red collagen and yellow/brown muscle cells.

7.3. Results

To exclude the possibility that the maintenance of a tissue weld was as a result of fixing the porcine splenic arteries in formalin, a number of frozen sections were prepared. These samples were deep frozen in liquid nitrogen and cut while frozen. This process avoided the lengthy, but relatively inexpensive preparation described above. However, it is a laborious and costly technique, and as a consequence is only used when obligatory.
In general, for both sets of specimen preparation, either frozen section or formalin fixed, there was evidence of good adhesion of the cut edges of the arteries, with a minimal amount of thermal injury. In no experiment, (even when using a laser power of 0.3 watts where the blood vessel showed obvious signs of charring on the outside), was thermal damage recognisable deeper than the thick tunica media (see figure 7.1). On a number of occasions the cut edges of the arteries were overlapped so that only the apposing media were glued (see figure 7.2), however in a number of examples the apposed vessels had slightly rolled over as a result of slight thermal injury. Although the apposition was not perfect, the vessels were glued (see figure 7.3). The similarities in results between the frozen and formalin fixed samples made it reasonable to assume that precipitation of the blood vessel proteins as a result of fixation played no part in the adhesion of the vessel edges. In a number of experiments there were plugs of adhesive within the lumen of the arteries, (see figures 7.4 and 7.5). The significance of this finding is unknown in this in vitro study. The results of bursting pressure tests revealed that anastomoses could be prepared that were able to withstand pressures in excess of the typical intraluminal pressures found within the body. However it is difficult to determine how the adhesion of the vessels took place. The cut edges were apposed and a layer of glue was visible between them (see figure 7.6), but it is impossible to speculate on how, and what the glue was bonding. Furthermore these histological experiments were unable to indicate the durability of the glue in vitro.
Figure 7.1: A histological section through a laser anastomosis prepared using an Eosin Y laser activated adhesive, without EDTA, irradiated at a power of 0.25 watts. Elastic van Gieson stain x 40.
Figure 7.2: A histological section through a laser anastomosis prepared using an Eosin Y laser activated adhesive, with EDTA, irradiated at a power of 0.2 watts. Haemotoxylin and Eosin stain x 40.
Figure 7.3: A histological section through a laser anastomosis prepared using an Eosin Y laser activated adhesive, with EDTA, irradiated at a power of 0.2 watts. Haemotoxylin and Eosin stain x 40.
Media

Figure 7.4: A histological section through a laser anastomosis prepared using an Eosin Y laser activated adhesive, without EDTA, irradiated at a power of 0.2 watts. Elastic van Gieson stain x 40.
Figure 7.5: A histological section through a longitudinal arteriotomy repaired by laser, using a Basic fuchsin laser activated adhesive, without EDTA. Irradiated at a power of 0.2 watts. Haemotoxylin and Eosin stain x 40.
Figure 7.6: A histological section through a longitudinal arteriotomy repaired by laser using a Basic fuchsin laser activated adhesive, without EDTA. Irradiated at a power of 0.2 watts. Haemotoxylin and Eosin x 16.
Conclusions and recommendations for future work.

At the outset of this project the fundamental objective was to define a clear end-point for surgeons during the laser irradiation of tissue. It was also hoped that we would be able to understand in more detail exactly how the laser radiation in combination with various biocompatible adhesives are bonding tissue together. The work described in this thesis has progressed from initial experiments investigating the interaction of laser radiation with biological tissue, through to the enhancing of laser radiation by suitably absorbing chromophores, and culminating with an extensive trial of in vitro experiments involving the laser repair of end-to-end anastomoses. The final selection of chromophore was particularly important with regards to their possible toxic and carcinogenic effects, any permanent staining of the tissue, and the risk of any adverse inflammatory reactions which may affect healing. In comparison to previous investigations where a number of different laser wavelengths were assessed, the experiments described in this thesis were conducted using an Argon ion laser arrangement. The results obtained as a consequence of laterally resolved reflection measurements illustrated the extent to which laser radiation was widely distributed in tissue, and highlighted the need for suitable chromophores to concentrate the laser beam, reducing the risk of thermal damage beyond the point of irradiation.

The use of a suitably absorbing chromophore significantly improved the ease by which longitudinal arteriotomies and end-to-end anastomoses could be prepared in vitro. Chromophores were invaluable when preparing patent blood vessel repairs quickly. In addition to the use of chromophores, proteins such as albumin and fibrin were combined with the chromophores, producing a 'laser activated adhesive'. It was hoped that these solutions would behave very much like a solder, acting as an efficient energy sink and enhancing the strength of the tissue bond.

When the Argon laser was used with chromophore and albumin for longitudinal arteriotomy repair in vitro, the resultant welds were more consistent than those repairs performed without chromophore or albumin. Burst pressure results from vessels repaired using the laser activated adhesive revealed that the optimal weld was
a least five times stronger than the comparative 'no chromophore' repair, and that these arteriotomies were able to withstand pressures beyond the normal physiological range. The optimum strength for arteriotomy repair with the laser activated adhesive was achieved using a laser power of 0.25 watts, an irradiation duration to end-point of 5 seconds and a spot size of 1mm. As the laser power was increased to 0.3 watts surface carbonisation was readily observed, which often resulted in premature failure.

The principle objective of this initial in vitro study was two fold. Firstly to gain experience of a highly specialised surgical technique with which I had no previous experience, and secondly to assess the possibility of using a combination of chromophore and albumin to reinforce or primarily repair the arterial incision. Further in vitro studies involved the fashioning of end-to-end anastomoses. As with previous studies, the use of chromophore and albumin increased the burst pressure strength and improved the consistency of the welding process. Burst pressure results from vessels repaired using the laser activated adhesive revealed that the optimal anastomoses were ten times the strength of the 'no chromophore' group. Many of these anastomoses were able to withstand pressures in excess of 500mmHg. In certain instances the anastomoses remained intact and pressure testing was stopped when the vessel began to leak from side branch holes or holes created by the stay sutures. The strongest end-to-end anastomosis prepared with chromophore and albumin was obtained using exactly the same conditions as those applied for the repair of longitudinal arteriotomies. It was observed that surface carbonisation was commonplace as the laser power was increased beyond 0.3 watts. Burst pressure testing revealed that these anastomoses invariably failed in charred regions. Histological assessment illustrated that although there was occasional evidence of slight thermal damage, the injury was superficial and the media and further internal layers of the vessel were unaffected. Repairs were effected using a minimal number of stay sutures, typically two. On average the total operating time, without any assistance from a technician, including vessel drying and cleaning, insertion of stay sutures and irradiation was of the order of 4 minutes, although the actual irradiation time was approximately 1-1.5 minutes. An anastomotic time of 1 minute would be a considerable advantage to a
surgeon attempting a multiple bypass operation. In some cases it may even remove
the need for the patient to be placed on heart lung machine
Although the results obtained from the pressure testing of end-to-end anastomoses
were encouraging, yielding vessel repairs able to withstand pressures well in excess of
the physiological range, at certain power ratings, and irradiation durations, some
degree of vessel carbonisation was unavoidable. Consequently a range of experiments
were performed involving the attempted photobleaching of two different
chromophore systems. Steady state irradiation and flash photolytic studies were used
to determine the quantum yields of fade of the dye/reducing agent systems. This
work culminated in a system based upon an Eosin Y/EDTA photoreduction. The
concentrations of dye and reducing agent were varied so as to give the desired
quantum yield of fade necessary to eliminate the possibility of over irradiating the
blood vessel. The suitability of this photofading 'laser activated adhesive' was
assessed by preparing further end-to-end anastomoses. The burst pressure of repairs
effected without reducing agent were compared to those prepared with EDTA. Each
group of vessels was able to withstand pressures well in excess of typical intraluminal
pressure. For vessels repaired using a photoreducible chromophore/albumin mixture
it was unnecessary to irradiate to an end-point since the fading of the chromophore
made it impossible to over expose the anastomosis. Macro- and microscopic
histological examination of these welds showed no evidence of thermal injury.
Conversely, anastomoses fashioned using a standard laser activated adhesive were
irradiated to a recognisable end-point. At high laser powers the irradiation times to a
glowing of the tissue were as short as half a second. Under such circumstances
control of tissue temperature was difficult and invariably led to thermal damage.
It is difficult to speculate by what mechanism the laser activated adhesive is
performing laser bonding. Coagulation of the tissue itself, in addition to that of the
albumin/fibrin mixtures is of particular importance, but was not the primary method by
which these anastomoses were performed. However, with regard to how and to what
the adhesive is bonding is a difficult question. Histological examination revealed that
the glue formed 'a plug' between the apposed arteries, and that there was direct
contact between the media and the adhesive. The extensive *in vitro* work has suggested that the ‘laser activated adhesive’ able to indicate an end-point for laser welding has considerable potential for a number of surgical procedures, and is not limited to the anastomosis of coronary arteries. Of particular interest would be its use in wound closure and haemostasis. Although promising results were obtained, the *in vivo* performance of the adhesive is unknown.

The primary aim of this thesis was to explore the mechanism of laser assisted vascular anastomosis, however it became evident from an early stage that the thermal alteration of the blood vessel walls was not producing the weld. The heating effects of the laser on the adhesive placed around the anastomosis were responsible for the union of the blood vessels.

Clearly there is a considerable amount of further work involved before the tissue glue described in this thesis could be used for clinical procedures. Initially the most significant task is to determine if simple anastomoses can be fashioned *in vivo*, that are able to sustain bursting pressures in excess of the normal physiological range. Furthermore, knowledge of the tensile strength of the repaired vessel would be important for determining the suitability of the laser activated adhesive for applications where the dynamics of the repair are significant. The bursting pressure tests presented in this study concentrated on the repair of longitudinal arteriotomies and end-to-end anastomoses. Although end-to-end anastomoses are performed during surgery, a more common procedure is end-to-side anastomosis. The patency of such anastomoses should be evaluated in further *in vivo* investigations. Histological investigation of these welds at regular intervals up to 30 days following repair would be particularly valuable in determining the short term behaviour of the adhesive with respect to its durability, possible foreign body reactions, if healing is possible through the adhesive plug, whether endothelial cells can regenerate on the surface of the glue, and the adhesive’s toxicity. Furthermore, electron microscopy scans would be of value in assessing exactly what parts of the tissue are adhering to
Conclusions and recommendations for future work.

The laser repair of blood vessels is a thermal process, with the laser acting as an accurate source of heat energy. For vessel repair using the Argon ion laser, red coloured chromophores are desirable. However the use of such solutions in vivo may not be suitable since the presence of blood within small vessels and on the surface of tissue may led to inadvertent coagulation and thermal injury to adjacent areas. Therefore there may be merit in exploring the use of other laser wavelengths, such as those emitting radiation in the red/orange region of the spectrum. The corresponding chromophores for this laser would be coloured green/blue, a more suitable choice for use within the body. During in vitro end-to-end anastomoses the basic recipe of the laser activated adhesive was altered slightly by the addition of bovine fibrin. However, if as suggested the glue is to be used for a range of different applications it would be of interest to explore the possibility of varying the basic glue recipe with regard to consistency, viscosity, pH, buffering, further biocompatible protein materials, EDTA and vitamin C, and with or without the addition of constituents that would enhance the lifetime of the adhesive, by reducing the effects of the hosts' phagocytic action.

As an aside from the development of biocompatible tissue adhesives, I feel that it is important to continue research in the area of laser/tissue interactions, not only with a view to gaining a greater understanding of laser tissue welding, but for the wider use of lasers in photoablation, photodynamic therapy, and their use as surgical knives. Knowledge of the distribution of light, heat, and possible radicals are of particular importance. Thermochromic and photochromic materials could be used as probes for determining the distribution of heat and light within laser irradiated tissue. Furthermore, the affects of absorption of light by tissue with regards to the formation of potentially harmful radicals could be monitored by diffuse reflectance flash photolysis.
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