An investigation into the adhesion of alginate solutions to oesophageal tissue

Hannah Batchelor

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School of Pharmacy,
University of London
29-39 Brunswick Square
London
WC1N 1AX
ABSTRACT

Bioadhesive devices have been investigated within the pharmaceutical industry for the past twenty years as a technique to enhance drug bioavailability from localised delivery formulations. Bioadhesive entities have previously been targeted at many sites within the body. This study investigated the feasibility of a liquid bioadhesive system targeted at oesophageal tissue. Such a system has implications in the treatment of gastro-oesophageal reflux disease and also as a novel means of localised drug delivery. Gastro-oesophageal reflux occurs when the acidic contents of the gastric material damage the oesophageal epithelium during reflux episodes. An adhesive alginate layer may enhance the protective systems present within the oesophagus and limit the damage caused by refluxate. Alginates are currently utilised in the management of gastro-oesophageal reflux disease and have also previously been shown to possess bioadhesive potential. This study investigated the interaction between alginate solutions and oesophageal tissue using techniques that allowed assessment on both a macro and microscopic scale.

The presence of mucin within the unstirred water layer resident on the oesophageal tissue surface was determined and quantified. The concentration of mucin within the layer was found to be 0.3 % w/v. Rheological studies investigated the interaction between alginate and mucin as well as interactions between alginate and physiologically relevant biological substrates. Rheological synergy is indicative of an adhesive interaction. Synergy was observed in mixed systems of alginate and components of the unstirred water layer. The physico-chemical properties of the alginate were not found to influence the extent of synergy observed.

The extent and duration of alginate adhesion on oesophageal tissue was assessed using two techniques; a retention model and microscopy. The results showed that alginate can adhere to oesophageal tissue for periods of at least 60 minutes. The physico-chemical properties of the alginate were not found to influence the retention of the alginate dose.
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CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

This study investigates the adhesion of aqueous solutions of alginates to œsophageal tissue. Such a system has implications as both a drug delivery tool and as a means of enhancing œsophageal defence against reflux of the stomach contents. Although the concept of bioadhesion was introduced into the pharmaceutical industry in the 1980's (Park & Robinson, 1984), very little work has been performed using the œsophagus as the site of adhesion. It is also worth noting that the adhesion of liquid formulations has been under-investigated. The introduction to this project will concentrate on the œsophagus as a site of adhesion, the basic concepts underpinning bioadhesion, especially the adhesion of a liquid formulation, and relevant aspects of alginate chemistry. Alginates are currently used within pharmaceutical formulations in the treatment of gastro-œsophageal reflux disease. Within these formulations the alginate moiety acts to form a raft that floats on the gastric contents thus physically limiting reflux. Alginate adhesion to the œsophageal mucosa may provide a secondary mechanism of treatment in gastric reflux disease.

1.2 THE OESOPHAGUS

The term œsophagus comes from the Greek word oisohagos. The word is made up from two parts, oiso which is the future tense of phero meaning to carry and phagein meaning food. The American spelling is esophagus, although, the English spelling will be used throughout this thesis. The œsophagus is a hollow muscular tube expanding from the pharynx to the stomach with a sphincter at either end. Its primary function is to carry food from the mouth to the stomach. Within this research project the œsophagus is the biological substrate that is the site of adhesion of an alginate formulation. In gaining an
understanding of the anatomy, physiology, histology and function of this organ, the mechanism behind the adhesion can be probed.

1.2.1 Anatomy of the œsophagus

The œsophagus is located behind the trachea and heart and in front of the spinal column; it passes through the muscular chest diaphragm before entering the stomach. Both ends of the œsophagus are closed off by muscular constrictions known as sphincters. At the anterior, or upper, end is the upper œsophageal sphincter, and at the distal, or lower end is the lower œsophageal sphincter. The anatomical location of the œsophagus is shown in Figure 1.1.

**Figure 1.1.** Schematic representation of the œsophagus position in humans (Barton, 1997)
The length of the human œsophagus in vivo has been determined (Li et al, 1994), the mean œsophageal length in 212 individuals being 23 cm with a range from 17-30 cm. The diameter of the œsophagus has been reported to be between 1.5 - 2cm (Mercer & Hill, 1988), although in vivo this organ exists as a collapsed tube. The important anatomical structures that allow the œsophagus to function effectively are the upper and lower œsophageal sphincters, the muscular body of the œsophageal tube and the posterior fixation of the œsophagus. Each of these anatomical features will be discussed individually.

1.2.1.1 Upper œsophageal sphincter

The muscular elements of the upper œsophageal sphincter are striated muscle, the cricopharyngeus as well as the adjacent portion of the cervical œsophagus and the inferior pharyngeal constrictor (Lang & Shaker, 1997). All these muscle components contribute to efficient sphincteric function. The sphincter is located at the position where the pharynx and œsophagus meet, notably the narrowest part of the œsophageal tube, level with the cricoid cartilage. The sphincteric muscle body forms a continuous muscle ring that acts to open and close under voluntary control. Relaxation and contraction of the upper sphincter initiates the primary peristaltic wave that propels material down the œsophageal tube.

1.2.1.2 Lower œsophageal sphincter

The existence of the lower œsophageal sphincter was the subject of debate for some time (Mercer & Hill, 1988). It was initially considered to have only a physiological role with no corresponding defined anatomical structure. However, the existence of the lower œsophageal sphincter has since been proven (Leibermann-Meffert et al, 1979). This muscle band, unlike the pyloric sphincter, which is short and thick, is an elongated thickening of the œsophagus above the gastric junction. The lower œsophageal sphincter is located partly in the abdomen and partly in the haital canal formed by the right crus of the diaphragm (Mittal, 1993).
1.2.1.3 Body of the oesophagus

There are two different muscle groups that extend the length of the oesophageal tube; inner circular muscle and outer longitudinal muscle. In fact, both muscle layers exist in a spiral formation around the oesophagus; the inner muscular layer is wound tightly and appears to have virtually circular windings and the outer layers are slowly unwinding and appear almost longitudinal. The upper third of the oesophageal body's muscle is striated muscle that is under voluntary control. The lower two thirds of the oesophagus is smooth muscle that is controlled involuntarily (Hopwood, 1984). The lower end smooth muscle present in the oesophagus is continuous with the smooth muscle of the stomach.

1.2.1.4 Posterior fixation of the oesophagus

Posterior fixation of the oesophageal body acts to ensure the oesophagus remains in its ideal location within the thoracic cavity. The lower oesophageal sphincter is held beneath the diaphragm and the gastro-oesophageal junction is held within the abdomen. The lower end of the oesophageal tube is fixed posteriorly to the pre-aortic fascia by the phreno-oesophageal ligament (Mercer & Hill, 1988). The large peristaltic forces can only act efficiently due to the fixation of the oesophageal tube.

1.2.2 Physiology of the oesophagus

The control and action of different components within the oesophageal tube enable the passage of food, which is the main function of the oesophagus, to occur effortlessly. Both the upper and lower oesophageal sphincters as well as the oesophageal body have a physiological role to play. The physiological role of these individual components will be described briefly.

1.2.2.1 Upper oesophageal sphincter

The upper oesophageal sphincter acts to seal off the oesophagus to the external environment. The striated muscle segment that makes up the sphincter is maintained
in a state of rest by constant discharge of motor action potentials. During swallowing these action potentials are abolished and relaxation occurs (Asoh & Goyal, 1978). A high resting pressure at the upper oesophageal sphincter is imperative as the negative resting pressure within the oesophageal cavity is approximately - 670 Pascals (Castell, 1988). This value is somewhat lower than normal atmospheric pressure, 101,325 Pascals. The high pressure at the upper oesophageal sphincter acts to prevent the oesophageal tube filling with air during the normal respiratory cycle. During the voluntary act of initiating a swallow the upper oesophageal sphincter relaxes leading to a co-ordinated sequence as forceful pharyngeal contractions move ingested material from the pharynx into the upper oesophagus. Respiration is inhibited during this process.

1.2.2.2 Lower oesophageal sphincter

The lower oesophageal sphincter is a 3-5 cm long muscle section that is continuous with the circular muscle of the inner oesophageal tube (Edwards, 1984). The lower oesophageal sphincter remains closed except during swallowing, vomiting and belching. The physiologic function of the sphincter is regulated by a complex interaction of three factors: sphincteric smooth muscle, autonomic and enteric innervation and the presence of gastrointestinal hormones and peptides. Resting tone within the oesophagus is approximately - 670 Pascals (Castell, 1988), which is a very different value than the pressure of + 670 Pascals found within the stomach. The relative differences in pressure prevents reflux of the stomach contents into the oesophagus.

1.2.2.3 Body of the oesophagus

Peristalsis and the motion of swallowing are physiological functions associated with the body of the oesophagus. Both the upper and lower oesophageal sphincters are also involved in this event. The swallow is often divided into three parts; the voluntary or oral stage, the pharyngeal or involuntary stage and the oesophageal stage (Edwards, 1984). The initial oral stage occurs when a bolus is collected within the oral cavity and manoeuvred to the posterior part of the mouth. The middle and inferior pharyngeal constrictors propel the bolus through the pharynx and towards the
oesophagus. At this point the swallow is no longer a voluntary action. At the pharyngeal stage the bolus enters the oesophagus via the upper oesophageal sphincter. The upper oesophageal sphincter relaxes and contraction of the superior constrictor muscle of the pharynx initiates a peristaltic wave propelling food into the oesophagus. The final or oesophageal stage is characterised by transport of the bolus by normal peristalsis the entire length of the oesophagus.

The peristaltic wave usually takes approximately 7-10 seconds (Castell, 1988). Normal peristaltic motion, superseded by a swallow is referred to as primary peristalsis. Secondary peristalsis is a similar wave that originates in the oesophagus and is thought to be the results of oesophageal distention produced by an ingested bolus or by gastro-oesophageal reflux (Meltzer, 1989). The physiological control mechanisms that govern the striated and smooth oesophageal musculature within a peristaltic wave are distinct. The striated muscle of the oesophagus receives exclusively excitatory vagal innervation and the peristaltic contraction of this segment results from the sequential activation of motor units in a craniocaudal sequence. Vagal control of the smooth muscle oesophagus is more complex than that of the striated muscle. Vagal fibres synapse on myenteric plexus neurons rather than directly at neuromuscular junctions and this type of vagal stimulation can either excite or inhibit oesophageal muscle depending on the stimulation parameters used (Edwards, 1984).

1.2.3 Histology of the oesophagus

Oesophageal tissue contains the four typical layers of the alimentary canal; mucosa (or mucous membrane), submucosa, muscularis, and tunica adventitia (Geboes, 1994). The mucosa is made up of stratified squamous epithelium containing numerous mucous glands. The submucosa is a thick, loose fibrous layer connecting the mucosa to the muscularis. The submucosa contains nerve fibres, large blood vessels, lymphatics and oesophageal mucous glands (Russell, 1988). These mucus glands are located in a longitudinal arrangement. The mucosa and submucosa form long longitudinal folds so that a cross section of the oesophagus opening is star-shaped. Figure 1.2 shows a labelled cross sectional view of the oesophagus.
The oesophageal mucosa consists of nonkeratinised, stratified squamous epithelium as a continuation of the epithelium present in the pharynx. Figure 1.3 shows the histological components within the oesophagus.

The luminal side of the oesophagus is lined by mucosa composed of epithelium, lamina propria and muscular mucosae. The different cell layers described in the squamous epithelium are the result of the processes of cell proliferation, differentiation, maturation and cell death. The oesophageal epithelium is a dynamic cell population that is renewed continuously. The tissue turnover time (time taken for the entire epithelium to be regenerated) has been estimated to be approximately 2 weeks (Geboes, 1994). Resident on top of the epithelial layer of the oesophagus is an unstirred water layer.
1.2.4 Functions of the œsophagus

The main function of the œsophagus is transport of food from the oral cavity to the stomach. A secondary function lies in the control of physiological reflux. Both functions will be discussed within this section. Oesophageal disease can lead to disturbances of either of the functions.

1.2.4.1 Transport within the œsophagus

Transport within the œsophagus requires co-operation of the three major structures associated with the œsophagus as an organ, namely the two sphincters and the œsophageal muscular body. These three parts act in synchrony to permit and promote smooth progression of the bolus from mouth to stomach. Oesophageal transit occurs as a swallow initiated procedure followed by a peristaltic wave that transports the bolus down the length of the œsophagus. The approximate speed of this peristaltic wave is $3.0 - 3.5$ cm/s (Humphries & Castell, 1977). Failure of transport can occur due to obstruction or malfunction of either sphincter or peristaltic
Chapter One: Introduction

function. The prominent clinical manifestation associated with oesophageal blockage is termed dysphagia.

1.2.4.2 Oesophageal defence systems in the control of reflux

Gastric reflux is a physiological event that occurs in all individuals. It is reported to occur more frequently after meals (Ippoliti, 1994). During the day the intraoesophageal pH is less than 4 for about 2% of the recording time in healthy individuals compared to the standard near neutral pH of 7 (DeMeester et al., 1976). Gastric reflux is the term used for the upward motion of the stomach contents travelling into the oesophagus. A primary aim of this research project is in protection of the oesophageal mucosa against gastric reflux, thus the mucosal defence systems involved will be discussed in further detail. Two important features associated with oesophageal defence are the lower oesophageal sphincter and the inherent epithelial resistance. Failure in either of these systems may result in the refluxed material damaging the oesophageal mucosa. In this light it is important to draw attention to the way in which these components prevent oesophageal damage.

1.2.4.2.1 The lower oesophageal sphincter (LOS)

The function of the lower oesophageal sphincter lies in the prevention of reflux of the gastric contents into the oesophageal cavity. The resting pressure at the sphincter is maintained at a sufficiently high level, compared to the pressure within the stomach, to prevent this event occurring. However, at times the sphincter is open, for example, during a swallow, when acting as a ventilation system for gaseous build-up within the stomach (belching) and during vomiting. Reflux occurs during periods of reduced lower oesophageal pressure, termed transient relaxations. Physiological reflux nearly always follows a transient relaxation of the LOS (94% of cases) although this mechanism is only responsible for 65% of the episodes of reflux in patients with gastro-oesophageal reflux disease (Dodds et al., 1982). The mechanism responsible for the induction of transient LOS relaxations is unclear. Incomplete swallowing has been suggested as one option (Mittal & McCallum, 1988), although other authors believe the relaxations are due to vagovagal reflexes initiated by
stimulation of mechanoreceptors in the gastric wall (Holloway et al, 1985). The incidence of transient lower oesophageal sphincter relaxations has been shown by some authors (Dodds et al, 1982; Dent et al, 1988) to be greater in patients with reflux disease than healthy individuals, although other work has suggested that the frequency is the same in both control subjects and those with gastro-oesophageal reflux disease (Pehlivanov et al, 1999; Mittal & McCallum, 1988).

1.2.4.2.2 The oesophageal mucosa

In instances where the lower oesophageal sphincter has failed to prevent gastric reflux the oesophageal mucosa is exposed to the refluxed material. The mucosal layer has inherent systems in place that aid in protecting the tissue from damage that may be caused by this refluxed material. The initial mechanism involves luminal clearance of the acid. This action removes the potentially damaging material and also limits the duration of exposure. Luminal clearance methods include the washing action of swallowed saliva and peristaltic movements clearing the length of the oesophagus. A study by Helm et al (1984) showed that nearly all the volume of refluxed acid was cleared by one or two peristaltic sequences. The residual acid was then neutralised by swallowed saliva in a stepwise fashion.

The inherent tissue resistance possessed by oesophageal mucosa can be broken down into three levels; pre-epithelial, epithelial and post-epithelial. Post epithelial defences are only involved if there is severe exposure to acid. The oesophageal blood supply acts as the post epithelial defence in removing excess carbon dioxide that may be produced during the neutralisation of hydrogen ions. It is also this blood supply that provides oxygen and nutrients that aid in cell repair if the cells should be damaged due to reflux exposure. A study by Hollwarth et al (1986), showed that the oesophageal blood flow increased during acid exposure.

Epithelial cells themselves are the next line of defence as the cell membranes and tight junctions slow down the process of acid diffusion into cells. The acid that does enter these cells is neutralised by protein, bicarbonate and phosphate within the cells. These neutralising components are delivered to the cells via the oesophageal blood
supply. Ion exchange channels present on the cell membranes also rebalance the intracellular pH by exchanging intracellular H$^+$ ions with extra-cellular Na$^+$ or by introducing HCO$_3^-$ from the cell exterior with Cl$^-$ ions from within the cell (Tobey et al, 1993).

The pre-epithelial layer is the first barrier that the refluxed materials make contact with, thus the defences in this area are the most vulnerable. Resident on top of the oesophageal epithelial layer is an unstirred water layer, although notably very little information is available on the characterisation of this layer. However, this layer, along with salivary secretions acts as the pre-epithelial defence system within the oesophagus and requires further discussion in this section.

The unstirred water layer has been estimated as being approximately 30 μm thick (Attwood, 1994) although other studies suggest a larger barrier of 95 ± 12 μm using dark field inverted microscopy techniques (Sarosiek et al, 1993). Studies of the pH of the unstirred layer using microelectrodes have been performed (Quigley & Turnberg, 1987). The measurements show a luminal to cell surface pH gradient of pH 3.2 - 4.8, which is not consistent with the normal luminal pH of 6.0 in the oesophagus (Edwards, 1984). When considering this layer as a defence system to refluxed gastric contents it is interesting to compare the pre-epithelial barriers in the oesophagus to those barriers present in gastric tissue. Figure 1.4 is a schematic representation of the defence systems present in the oesophagus compared to those present in the stomach.

Although the difference in thickness between the two defensive layers is not shown in Figure 1.4, the structural differences are highlighted. No continuous mucus layer is present on oesophageal tissue. This accounts for the difference in pH gradient observed from the luminal edge to the tissue surface. The mucus barrier is able to slow down the diffusion of acidic components thus minimising damage that may be caused to the cellular tissue. Hydrogen ions can easily permeate the unstirred water layer resident on oesophageal tissue in comparison to the gastric mucus layer. Neutralising bicarbonate ions are transported from the underlying blood supply to the gastric tissue surface via tight junctions. This system is more effective than the diffusion system present in oesophageal tissue.
(a) The pre-epithelial defences of œsophageal epithelia.

(b) The pre-epithelial defences of gastric epithelia.

Figure 1.4. Schematic representation of pre-epithelial defence within the œsophagus and stomach. Adapted from Orlando et al (1994).

This diagram compares the defence systems of œsophageal and gastric epithelia. The pH gradient within each layer is compared as well as the aggressive and protecting chemical agents involved in the attack and defence mechanisms. The mucus layer present in the human stomach has been shown to be "always continuous yet of varying thickness" along the epithelial layer (Allen, 1989). The average thickness has been calculated as being 180 μm (Allen, 1989). The electrolytic composition of gastric mucus is similar to that of plasma. The mucus gel when removed from the surface contains 90-95% by weight water meaning that it is permeable to small molecules such as H⁺ ions and solutes (Hollander, 1963). Molecules including proteins and proteinases such as pepsin are too large to easily permeate the mucus layer, thus their rate of diffusion through this layer is relatively slow. The mucus layer in the stomach is under constant renewal and is therefore a
dynamic entity. As the mucus is secreted from goblet cells it moves from the cellular surface to the exterior of the adhered layer where it is degraded by enzymes or eroded away due to mechanical shear. The passage of mucus in the direction from the cell surface to the lumen also hinders the opposing penetration of larger molecules thus preventing them from damaging the gastric cell layer. The gastric mucus layer can uphold a pH gradient of 3-4 pH units (Quigley & Turnberg, 1987).

In contrast to this, the unstirred water layer resident on the oesophageal epithelium has been shown to be very much thinner, in the range of 30 - 95 μm (Attwood, 1994; Sarosiek et al, 1993). The pH gradient of the unstirred water layer is also much smaller with a difference of only 1 pH unit from the luminal side to the cellular surface (Orlando, 1994). As mentioned previously, the composition of the unstirred water layer has not been well characterised. It is considered to consist of swallowed saliva components and material secreted from the oesophageal submucosal glands. The composition will be investigated further in this introductory chapter as it is an important factor in the adhesion of an alginate solution to oesophageal tissue.

1.2.4.2.3 Composition of the unstirred water layer

Although this resident protective barrier is termed an unstirred water layer, it is not comprised of water alone. Submucosal glands present in the oesophageal epithelium secrete many substances that may be represented within this layer. Materials from swallowed saliva may also be present within this layer.

Submucosal glands secrete directly into the oesophageal lumen and it is from this site that fluids have been collected and assayed to determine the specific secretory products of the glands. It was shown that the submucosal glands secrete water, electrolytes (including bicarbonate ions), mucin, epidermal growth factor and prostaglandins (Long & Orlando, 1999). These secretory products play a role in protection of the oesophagus. Bicarbonate ions act to neutralise any acid that is refluxed into the oesophageal cavity, while mucins aid in building a gel-like layer that defends against larger molecules such as pepsin via a physical diffusion barrier. Myers et al (1992) examined the secretion of bicarbonate from human submucosal
glands *in vivo*; both vagal stimulation and œsophageal acid perfusion were found to stimulate secretion of bicarbonate ions. A second source of bicarbonate ions present in the unstirred water layer may be derived from swallowed saliva. The secretion of mucin from the œsophageal submucosal glands has been investigated (Namiot et al, 1994a). The results show that mucin is released at a rate of $0.23 \pm 0.03 \text{ mg cm}^{-2}\text{min}^{-1}$ this rate being relatively constant for a period of approximately 8 minutes. The authors analysed the material present in the œsophageal lumen and assumed it to be derived from œsophageal secretions. They also suggested that the mucin detected was structurally distinct from both salivary and gastric mucin. Their study used a Periodic acid-Schiff (PAS) assay that detected and quantified the presence of glycoprotein as an indication of mucin levels (this analysis method is described in further detail in Chapter 3). However, it should be noted that there are many other PAS positive substances present in human œsophageal epithelium (Long & Orlando, 1999).

Saliva is an important component of the unstirred water layer resident on œsophageal tissue. It has been suggested that salivary secretion quantitatively and qualitatively contributes to the protective potential of the pre-epithelial barrier (Sarosiek & McCallum, 1995). Saliva contains phosphate, calcium and bicarbonate ions that act as buffering agents, thus the acid neutralising capacity of this fluid can aid in restoration of the œsophageal pH towards the neutral state. In addition to its neutralising capacity saliva also contains growth factors that have cytoprotective and healing properties in various segments of the gastro-intestinal tract. Growth factors are polypeptides that can enhance the body's repair mechanisms after tissue injury. The ability of swallowed saliva to adhere to the œsophageal surface and interact with the unstirred water layer is believed to be due to the presence of salivary mucins (Mellema et al, 1992). It has been suggested that due to the rheological profiles of salivary mucins they are able to concentrate themselves onto the oral mucosa forming an effective barrier against dehydration and environmental insult (Tabak et al, 1982). This rheological phenomenon may also be extended into the œsophageal cavity. Mucins are glycoproteins that give the typical visco-elastic character to all mucosal secretions. General functions of mucins include cytoprotection, lubrication and moisture retention. The capacity of mucins to protect epithelial surfaces depends largely on their structure and their ability to form a gel layer together with other
salivary components. The rheological properties of mucins are the result of non-covalent intermolecular interactions between the polymeric mucin molecules and are not just the result of intermolecular entanglements (Schwarz, 1987). Salivary mucin is considered to be biochemically and physically similar to gastric mucin and may therefore protect the œsophageal mucosa against attack from hydrogen ions and pepsin (Kongara & Soffer, 1999).

Thus the protective nature of saliva in œsophageal defence has two major parts; its neutralising capacity and its ability to concentrate itself on œsophageal mucosa and act as a defence barrier to limit the penetration of H⁺ ions and pepsin. Studies performed have shown that the volume of saliva output (Namiot et al, 1994b) and also the volume of mucin output (Sarosiek et al, 1994) are increased during chemical stimulation of the œsophagus. Saliva is therefore acting to enhance the pre-epithelial defence systems present in the œsophagus.

1.2.5 Diseases of the œsophagus

Oesophageal disease is usually the result of œsophageal malfunction. Oesophageal blockage leads to an inability of the organ to transport food from the mouth to the stomach. Depending on the nature of the blocking agent the œsophageal mucosa may also be susceptible to damage at the site of blockage. Lodging of tablets has been linked with local œsophageal injury (Kikendall et al, 1983).

The reflux of gastric contents into the œsophagus occurs intermittently to everyone at certain times; the type of reflux that causes neither symptoms nor histological change is referred to as physiological gastric reflux. Gastro-œsophageal reflux that causes symptoms or complications is called pathological gastric reflux and may lead to gastro-œsophageal reflux disease (GORD) (Jamieson & Duranceau, 1988). Gastro-œsophageal reflux disease is a well-characterised disease state that occurs when the frequency and/or duration of reflux episodes is increased. GORD is an all-encompassing term that is used to describe the full spectrum of disease resulting from acid exposure within the œsophagus (Locke, 1996). The most common symptom of GORD is retrosternal pain known as heartburn. The symptoms and
degree of oesophageal mucosal damage are primarily determined by the pH and concentration of the refluxate as well as the duration of oesophageal acid exposure (Orlando, 1991a).

Although reflux is a normal physiological event, it is not known how this translates to GORD. Many factors are thought to be involved and these will be discussed in further detail to explain the basic aetiology of this very common disorder. Effective functioning of the oesophagus in preventing damage by gastric reflux involves the lower oesophageal sphincter, oesophageal acid clearance and oesophageal epithelial resistance. A failure in any one of these components may lead to an inability to sufficiently protect the oesophageal mucosa against refluxate exposure and thus the development of GORD. Relaxation of the lower oesophageal sphincter allows reflux of the gastric contents to occur, thus ineffective control of the lower oesophageal sphincter is a major consideration in the causation GORD (Dodds et al, 1982). Certain drugs that reduce lower oesophageal sphincter pressure should be avoided as reduction in sphincter tone would exacerbate the reflux condition. Drugs that reduce lower oesophageal sphincter pressure include calcium channel blockers, nitrates, theophylline, benzodiazepines and antimuscarinics (Katz, 1991). Peristaltic dysfunction has also been linked to the causation of GORD as has a reduction in saliva secretion rate (Kharilas et al, 1988).

There are many techniques available in assessing gastro-oesophageal reflux disease. The severity of the symptoms are an indication of the terminology used in classification of the disease. The major complications of GORD are erosive/ulcerative oesophagitis, oesophageal stricture and Barrett's oesophagus. All of these complications result from the damage inflicted by gastric contents on the oesophageal mucosa and changes caused by subsequent repair and fibrosis (Peters & DeMeester, 1993). Oesophagitis is graded using endoscopy and well over 100 grading systems have been used in the literature. This extensive coverage has caused much confusion although two popular systems are shown in Tables 1.1 and 1.2. Figure 1.5 shows endoscopic images of complications associated with GORD.
All reflux-grading systems have advantages and disadvantages. The Los Angeles consensus system is thought to be less ambiguous and thus a more universally acceptable system to assess the damage observed.

**Table 1.1.** Savary-Miller system for grading oesophagitis (Savary & Miller, 1978)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Solitary (occasionally multiple) erythematous/exudative erosion, covering a single mucosal fold.</td>
</tr>
<tr>
<td>2</td>
<td>Multiple erosions covering several mucosal folds, partly confluent but never circumferential.</td>
</tr>
<tr>
<td>3</td>
<td>Circumferential extension of erosive lesions.</td>
</tr>
</tbody>
</table>
| 4      | a) Ulcer.  
         | b) Fibrosis, leading to stricture and brachyoesophagus. |
| 5      | Columnar metaplasia (islet, strip, sleeve). |

**Table 1.2.** Los Angeles system for classification of reflux oesophagitis (Tytgat, 1998)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>One or more mucosal breaks each no longer than 5mm.</td>
</tr>
<tr>
<td>B</td>
<td>At least one mucosal break more than 5mm but not continuous between the top of two mucosal folds.</td>
</tr>
<tr>
<td>C</td>
<td>At least one mucosal break that is continuous between the tops of two or more mucosal folds, but which is not circumferential.</td>
</tr>
<tr>
<td>D</td>
<td>Circumferential mucosal break.</td>
</tr>
</tbody>
</table>
Figure 1.5. Comparison of an endoscopic image of a (a) healthy oesophagus, (b) an oesophagus showing lesions and (c) Barrett's oesophagus. Images taken from: www.gerd.com/intro/frame/endoscop/htm.

The three images in Figure 1.5 show the differences in tissue seen in cases of reflux oesophagitis. Figure 1.5 (b) shows lesions running both longitudinally and circumferentially on oesophageal tissue. Barrett's oesophagus appears as a much redder colour within the oesophageal mucosa, shown in Figure 1.5 (c).

Structures associated with severe erosive oesophagitis develop from submucosal fibrosis. The size of strictures present in the oesophagus varies a great deal although the average lesion length is 1 - 2 cm long (Morton & Fromkes, 1993). Severe stricture can lead to dysphagia. Barrett's oesophagus is usually defined by the presence of columnar mucosa in the lower part of the oesophageal tube (Spechler & Goyal, 1986). This histological alteration is a result of extensive and prolonged oesophageal damage caused by gastric reflux. It is widely believed that almost all oesophageal adenocarcinomas develop in the columnar epithelial lining associated with Barrett's oesophagus (Hawe et al, 1973; Haggitt et al, 1978). Because columnar epithelium is more resistant to acid, this condition is associated with the alleviation of heartburn (Peters & DeMeester, 1993). One study (Spechler, 1989) showed that 25 % of patients known to have the oesophageal changes associated with Barrett's had no symptoms of reflux.
1.2.6 Treatment of GORD

The goals of treatment in GORD include relief of symptoms, healing and prevention of the relapse of mucosal damage and prevention of the complications associated with long term œsophagitis. There are three stages associated with the treatment of reflux œsophagitis; non-pharmacological treatment, pharmacological treatment and surgical management. These three stages are performed according to the severity and extent of the symptoms and/or damage present.

Non-pharmacological management of GORD includes lifestyle modifications that can aid in the treatment of this disease. These changes should limit the amount or the duration for which refluxed material is in contact with the œsophageal mucosa. These alterations include monitoring the diet to reduce obesity as well as limiting the intake of cigarettes and alcohol (Katz, 1991).

The aim of drug therapy (pharmacological management) is to decrease acid exposure to the unprotected œsophagus, reducing pain and allowing natural healing mechanisms to reduce inflammation. The first therapies used are antacids and alginate-antacid preparations. Prescription medications to treat GORD include histamine receptor antagonists (H₂ blockers), proton pump inhibitors (PPIs) as well as promotility agents which aid in the clearance of acid from the œsophagus. The current drug therapeutic agents will be discussed in further detail paying attention to their advantages and disadvantages.

1.2.6.1 Antacids

Antacids are used for mild disease and are available over the counter for self-administration. They are often used in association with other forms of therapy. Antacids are weak bases that interact with gastric acid to form a salt and water; this neutralisation of gastric acid also aids in increasing lower œsophageal sphincter pressure (Higgs et al, 1974). Sodium bicarbonate, calcium carbonate, aluminium hydroxide and magnesium hydroxide are the most common antacids in current use. Antacids have a short duration of action: 45-60 minutes during fasting, or 1 to 3 hours if administered 1 hour after meals (Garnett, 1993). Adverse reactions to
antacids often include an alteration in bowel habits, for example, aluminium hydroxide can lead to constipation and magnesium salts have a cathartic effect. Because they may interact with other drugs either by altering the rate or extent of absorption or the renal elimination of certain agents, antacids should be taken at least 2 hours before or after other medications.

Alginate-containing formulations, such as Gaviscon Liquid®, form a raft that floats on the stomach contents that impedes reflux and protects the oesophageal mucosa (Beckloff et al, 1972). Galmiche (1998) has shown that alginate-containing antacids are more effective than simple antacids for symptomatic relief. In more severe cases, the “raft” itself may be refluxed and may protect the inflamed mucosa allowing healing to occur (McKay et al, 1989). The damage caused by the acid associated with GORD is due only to the inappropriate location of the acid rather than hyperacidity. Therefore, raft formation restricts the location of the acid to within the stomach.

1.2.6.2 Histamine receptor antagonists

Since the mid 1970's, acid suppression agents, known as histamine (H2) receptor antagonists have been used to treat GORD. Until recently these drug were only available on prescription. H2 antagonists bind to the H2 receptors on gastric parietal cells resulting in inhibition of basal stimulated acid secretion (Goodman & Gilman, 1990). Conventional doses of these drugs can reduce the 24-hour acid secretion by 60-70 % (Aadland et al, 1977). Higher doses (800 mg twice daily) of H2 antagonists (for example, Cimetidine) are more effective than placebo in healing erosive oesophagitis (Palmer et al, 1990), although lower doses (400 mg twice daily) (Farup et al, 1990) are sufficient to relieve the symptoms of heartburn.

1.2.6.3 Proton pump inhibitors

Proton pump inhibitors (PPIs) block the parietal cell H+ - K+ ATPase "proton pump". These agents are the most potent inhibitors of gastric acid secretion available. Omeprazole was the first drug to be marketed within this group of agents. Multiple trials have all demonstrated superiority of proton pump inhibitors over H2 antagonists, sucralfate and promotility agents in the treatment of GORD (McTavish
Proton pump inhibitors have also been shown to aid in the endoscopic healing of reflux oesophagitis (Sontag et al, 1992) although long term treatment is required in order to prevent relapse (Boer & Tytgat, 1994). PPIs have been shown to be a more cost effective maintenance therapy than H$_2$ antagonists in the treatment of GORD (Welage & Berardi, 2000). Adverse effects of PPIs include headache, diarrhoea, constipation and nausea. As these drugs act to increase the gastric pH, the absorption of other medications may be affected.

1.2.6.4 Promotility Agents

Promotility drugs are effective in the treatment of mild to moderate symptoms of GORD. These drugs are also referred to as prokinetic drugs. Cisapride, a substituted benzamide is an example of a promotility agent. It acts to stimulate gastro-intestinal motility probably by increasing the release of acetylcholine in the gut wall at the level of the myenteric plexus (McCallum et al, 1988). These drugs increase lower oesophageal sphincter pressure, which helps prevent acid reflux and improves the movement of food from the stomach. They decrease heartburn symptoms, especially at night, by improving the clearance of acid from the oesophagus and stomach (Maddern et al, 1990). The value of prokinetic drugs in the treatment of GORD is not clear as adverse effects may be a problem. There are reports of benefits both with cisapride alone (Toussaint et al, 1991) and also with cisapride in combination with an H$_2$ antagonist (Galmiche et al, 1988).

1.2.6.5 Mucosal Protective Agents

This class of drugs includes sucralfate and colloidal bismuth compounds. Sucrose octasulfate, usually termed sucralfate is a cytoprotective agent that in certain conditions is capable of forming an adherent complex with proteins. Sucralfate has had only limited use in the management of GORD as the main aim of treatment is in the reduction of exposure of the oesophagus to acid. The results of studies investigating the role of sucralfate in the treatment of GORD have been inconsistent (Orlando, 1991b). Sucralfate can interfere with the bioavailability of a range of
drugs including tetracyclines, phenytoin, ciprofloxacin, digoxin, warfarin and sulpiride (Geising et al, 1983).

1.2.6.6 Surgical management of GORD

Antireflux surgery is reserved for patients with complicated GORD who will require lifetime medical therapy (Richter, 1992). Unlike surgery to remove a diseased organ whereby the organ will be destroyed with its removal, antireflux surgery is designed to improve the function of the organ. The most common antireflux operation worldwide is the Nissen 360 degree fundoplication. Other procedures include two partial fundoplications, the Belsey 270 degree and the Toupet 180 degree and the Hill posterior gastropexy (Peters, 1993). Each of these procedures involves the reduction of hiatal hernia, if present as well as construction of a valve system (Richter, 1992).

1.3 BIOADHESION

Adhesion of an alginate layer to oesophageal tissue has implications in assisting the inherent defence system of the oesophageal mucosa to protect against damage caused by refluxed gastric contents. Bioadhesion is a key feature of such a system and warrants further discussion here.

Bioadhesion is defined as the state in which two materials, at least one of which is biological in nature, are held together for an extended period of time by interfacial forces (Good, 1976).

In biological tissue, four types of bioadhesion can be distinguished (Gayot, 1985):

- Adhesion of a normal cell to another normal cell
- Adhesion of a cell with a foreign substance
- Adhesion of a normal cell to a pathological cell
- Adhesion of an adhesive to a biological substrate.
A bioadhesive has been defined as a synthetic or biological material that is capable of adhering to a biological substrate, e.g. tissue (Peppas & Buri, 1985). If an adhesive system attaches to a mucus coat the term mucoadhesion is used preferentially (Leung & Robinson, 1990; Park, 1989) and when the biological tissue involved is the stomach the term gastroadhesive may be used (Jordan, 1990). The concept of mucosal adhesives (mucoadhesives) was introduced into the pharmaceutical sciences in the early 1980's (Mortazavi et al, 1992). Sites for application of bioadhesive drug delivery systems include the eye, nasal cavity, buccal cavity, vagina, colon and the skin (Helliwell, 1993; Greaves & Wilson, 1993). Targeting a drug or a drug delivery system to a particular region of the body for an extended period of time has implications in controlling the activity of a drug. Locating the formulation at the site of adhesion minimises diffusion barriers and prolongs the residence time. Both these actions enhance the absorption of a drug.

Adhesion is an all-encompassing term that can be used to describe a wide variety of interactions. This introductory chapter will discuss bioadhesion, as this term encompasses both mucoadhesion and gastroadhesion. The term mucoadhesion may be used in reference to oesophageal adhesion due to the mucin present on oesophageal tissue.

In adhering an alginate solution to oesophageal tissue there are many factors that need to be addressed. These include, the nature of the oesophagus as a site of adhesion, which has been covered in the previous section. Secondly, the alginate-oesophagus interface needs to be investigated. Thirdly, the adhesion of a liquid system is very much under-investigated in the field of pharmaceutical bioadhesion. For this reason it is important to relate the current knowledge of adhesion of solids and gels to a liquid system. The theories of bioadhesion will be discussed as a means of characterising the nature of the adhesive interaction. Methods used to measure the strength of adhesion will also be discussed in relation to assessing the adhesive interaction. Finally bioadhesive systems in current use will be discussed briefly with special reference to liquid bioadhesive systems.
1.3.1 Theories of bioadhesion

There are many theories that aim to explain the fundamental mechanisms of adhesion. In any given system there may be more than one theory that can be applied to explain aspects of the adhesive interaction. Theories of bioadhesion enable the adhesive interaction to be probed. Theories highlighted in the literature include electronic, adsorption, wetting, interpenetration of polymer chains, diffusion and fracture theories (Ahuja et al, 1997). Each of these theories will be discussed here.

1.3.1.1 Electronic theory

This theory suggests transfer of electrons between two substrates leads to the formation of an electrical double layer at the interface (Deijaguin et al, 1977). The electron transfer occurs due to the difference in structure between the bioadhesive agent and the biological substrate. Adhesion occurs due to attractive forces across the double layer.

1.3.1.2 Adsorption theory

The adsorption theory suggests that the adhesive interaction is due to surface forces between the two components (Kinloch, 1980). After initial contact, two surfaces interact due to surface forces acting between the atoms in the two materials. The forces in this theory may be covalent, electrostatic, Van der Waal's, hydrogen or hydrophobic. This type of interaction is considered to be one of the strongest contributors to bioadhesion (Longer & Robinson, 1986).

1.3.1.3 Wetting theory

This theory is primarily of concern to liquid bioadhesive systems and is thus of particular relevance in this project. This theory examines the ability of a liquid or paste to spread over a biological substrate (Kaelbe & Moacanin, 1977). This theory uses analysis of the spreading coefficient of a liquid bioadhesive over a tissue by displacement of the surrounding fluid. The energy involved in the interaction
between the adhesive system and the biological substrate must be greater than the corresponding interactions between the tissue and surrounding fluid and also the adhesive liquid self-interactions.

1.3.1.4 Diffusion theory

The diffusion or interpenetration theory was proposed by Voyutskii (1963) and was adapted to refer to a polymer gel interface by Mikos & Peppas (1990b). According to the diffusion theory the biological substrate and adhesive material mix to a certain depth and this interaction leads to a semi-permanent bond. This theory has special relevance in this work as it refers in particular to polymeric adhesive systems interacting with mucus covered substrates. The depth to which the two components (mucus and polymer) interact is dependent on the diffusion coefficient and the time of contact. The diffusion coefficient depends on the physical properties of the adhesive polymer as both the molecular weight and cross-linking density have both been shown to have an effect (Reinhart & Peppas, 1984).

1.3.1.5 Fracture theory

The fracture theory relates the difficulty in separating two surfaces (after adhesion) to the bioadhesive strength. This theory is the basis behind many methods used to assess the strength of the bioadhesive bond. This theory depends upon the fracture occurring exactly at the substrate-tissue interface, although this has not always been shown in practice (Mikos & Peppas, 1986).

1.3.1.6 Swelling theory

This theory investigates the importance of water within the bioadhesive interaction. This factor is of great importance in solid bioadhesive formulations. Water from the biological substrate may be drawn out to interact with the bioadhesive formulation leading to a strong interaction. The polymer - water interactions supersede the corresponding polymer-polymer interactions and thus a strong bond is formed. Hydration of a polymer results in the relaxation of stretched, entangled or twisted
molecules. This enables adhesive sites to be exposed thus enabling them to interact with the biological substrate.

Overall bioadhesion can be separated into two processes. The first stage is a contact stage where the interaction is governed by surface energy effects and a spreading process. The second phase is determined by the structure and diffusion of the adhesive formulation, whereby the interface formed determines the strength of the final bond.

1.3.2 Methods used to assess bioadhesion

In testing bioadhesive formulations, the test used should be appropriate to the particular system under development. This means that, as well as adhering, the bioadhesive formulation should be able to perform its designated function be it as a drug delivery device or as a protective agent. The nature of the biological substrate should be considered in test development, for example, adhesion to gastric mucosa would require a very different test to that of a topical adhesive device. Physiological conditions need to be considered and mimicked to produce an optimum testing system. In drug delivery systems the main purpose of the bioadhesive system is to increase the efficacy of delivery of the drug in question. *In vivo* tests are the most appropriate situation used to reveal this required information. However, they are both costly and time consuming and for this reason the majority of information available on bioadhesive drug delivery agents comes from *in vitro* tests. These simpler, routine tests have been developed to allow many different bioadhesive formulations to be tested, thus acting as a screening mechanism for candidate bioadhesive agents. In most cases a simple test that is not influenced by physiological factors is more convenient than an *in vivo* bioavailability test. However, attempting to extrapolate data from such a test should be treated with caution, as the controlled environment may bear little relationship to the ultimate performance of the bioadhesive. Important considerations in the design of such tests include the residence time of the adhesive formulation and the adhesive interaction strength.
The main tests that have been used to monitor bioadhesion in vivo include gamma scintigraphy and transit studies with radiolabelled dosage forms. Pharmacokinetic data can be obtained in conjunction with these studies and represents the ultimate test for a bioadhesive drug delivery system. The limitations of the above test methods include the fact that the tests measure the retention or distribution of the dosage form yet not the clinical efficacy of the drug. Complexes that may form between the delivery matrix and drug must be accounted for and also the way in which the delivery system affects the release of the drug.

In vivo tests can provide quantitative information on the bioadhesive properties of a bioadhesive dosage form. Radioisotopes have been used to measure the GI transit time of several bioadhesives. Ch'ng et al (1985) labelled a bioadhesive with $^{51}$Cr and the time dependant distribution of the radioactivity in the gastrointestinal tract was measured. γ-scintigraphy has also been used to monitor bioadhesives labelled with radionuclides such as $^{99}$Tc, $^{113}$In or $^{125}$I.

In vitro tests were initially designed to screen potential bioadhesives with a view to in vivo testing. The in vitro tests performed now are often set up to provide information on the nature of the bioadhesive interaction and thus the ways in which the bioadhesive agent can be developed and enhanced.

Force of detachment tests are more commonly used tests available to assess the bioadhesive properties of many formulations. They are used to directly measure the forces involved in separating adhesive material from the tissue surface. Many different forms of this test have been described (Park & Park, 1990) although a lack of standardisation between test methods has given rise to non-uniform results. As these tests are so widely used in the field of bioadhesion their advantages and disadvantages will be discussed.

1.3.2.1 Force of detachment tests

The stress applied to assess the adhesive strength can be delivered in three different ways; tensile, shear and peel stress. When the force of separation is applied perpendicularly to the tissue/adhesive interface a state of tensile stress is set up.
During shear stress the direction of forces are parallel to the tissue/adhesive interface. In both shear and tensile modes the stress applied to the interface is uniform. Peel tests are more appropriate in measuring systems where adhesive tape may be involved. The peel test is of limited use in bioadhesive systems although it does have an application in the testing of transdermal patch technology.

A typical measuring system for tensile stress is seen in Figure 1.6; these tests can also be referred to as force of detachment tests. Detachment tests were the first methods used to measure bioadhesion \textit{in vitro}. Ishida et al (1981) measured the adhesion of a solid dosage form to mouse peritoneal membrane. Ch'ng et al (1985) and Park et al (1985) used the same type of system with rabbit stomach mucosa.

This test provides repeatable measurements although the tensile force may not always be the most appropriate considering the \textit{in vivo} nature of the drug delivery system. A bioadhesive delivery system is more likely to be dislodged in a parallel plane or peeled away from the mucosal surface.

\begin{figure}[h]
\centering
\includegraphics[width=0.3\textwidth]{schematic_tensile_stress}
\caption{Schematic of apparatus used to measure tensile stress}
\end{figure}

The shear stress measures the force that causes the bioadhesive to slide with respect to the mucosal surface in a direction parallel to their plane of contact. Figure 1.7 illustrates the flow channel method developed by Mikos & Peppas (1990a). This system measures the strength of the shear force required to detach a dosage form from a substrate.
Figure 1.7. Apparatus for the flow channel method

In this experiment humid air at 37 °C was passed over a particle of a mucoadhesive polymer placed on mucus gel inside the flow cell. Static and dynamic behaviour of the particle was monitored at frequent intervals using a camera. The experiment may also be conducted using a freshly prepared mucus layer or tissue.

Other methods used to assess bioadhesion focus on the retention of a formulation on the substrate rather than the forces involved in separating the tissue/adhesive interface. These tests can be grouped as retention models and will be further discussed in more detail below.

1.3.2.2 Retention Models

Retention models used include the perfused intestinal loop. This method was developed by Poemla & Tukker (1987) and has since been used in studies to measure bioadhesion. The method involves isolating a section of intestine that has an intact blood supply, thus mucus biosynthesis and secretion can still occur, this type of ex vivo section provides an ideal substrate with which to assess bioadhesion. Poly-2-Hydroxyethylmethacrylate (p-HEMA) microspheres coated with a bioadhesive agent were examined by Lehr et al (1990). These microspheres were perfused through the tissue and the effluent measured and analysed using a Coulter counter to determine the fraction of particles remaining in the loop.

The falling film technique described by both Teng et al (1987) and Rao et al (1989), used coated glass or latex particles that were dispensed onto the tissue surface. The tissue was then washed at a suitable rate with an appropriate medium. The effluent
was collected and analysed to detect how well the coated particles adhered to the mucosal surface.

Park & Robinson (1984) investigated fluorescently labelled human conjunctival epithelial cells. Addition of polymers to the substrate surface altered the fluorescence and thus the degree of bioadhesion could be measured.

Iooss et al (1995) developed a novel system to investigate the adhesiveness of gels as applied to a model oesophagus. The substrate used was flexible polyethylene tubing. Gels were prepared in fluorescent solutions and applied to the tubing at an angle. After a set period of time the apparatus was returned to the vertical and washed with water to mimic saliva flow. The effluent was collected and the fluorescence measured to determine the amount of gel removed from the substrate. This work was followed by in vivo work using isolated rabbit small intestine.

1.3.2.3 Rheological tests

As well as investigating the forces involved in the separation of the tissue/adhesive interface and the retention of the formulation, other methods are used that probe the interaction in a different manner. Rheological techniques are popular due to their simplicity and their relevance to the physiological environment. These tests assess the mechanical properties of a formulation; they can be investigated in combination with the physiological fluid present at the adhesive-tissue interface such as mucus or saliva. Forces such as chewing and swallowing can also be mimicked in the assessment of oral delivery formulations. Rheological tests are very useful when investigating the bioadhesive properties of gel like or liquid formulations and will thus be further discussed here.

Rheological evaluation of the interaction between various polymers and mucin has been used to assess the mucoadhesion of certain candidate bioadhesives. It is believed that an interaction that shows positive synergy between the polymer and mucin is indicative of a good bioadhesive agent (Mortazavi et al, 1992). Oscillatory (Caramella et al, 1992), flow (Hassan & Gallo, 1990) and creep (Rossi, 1999)
analysis have been performed on polymer-mucin mixtures to measure synergy between the components.

Having investigated the techniques available to assess bioadhesion and also the theories involved in the adhesive interaction, it seems sensible to investigate both the factors that make a formulation a "good bioadhesive" and also an overview of bioadhesive agents in current use. When looking at bioadhesive formulations a common factor is the presence of a polymeric carrier. This literature review will focus only on polymeric systems as this has the most relevance in a project investigating the adhesion of an alginate polymer on oesophageal tissue.

1.3.3 Bioadhesive polymers for pharmaceutical applications

Hydrophilic polymers, due to their large molecular size and ability to form intermolecular bonds, are ideal bioadhesive candidates. Both natural polysaccharides such as chitosan, cellulose, starch and alginic acid and synthetic polymers such as polyacrylic acid and carboxer can act as bioadhesive agents. These polymers can act as bioadhesive agents in three different ways; those that become sticky when placed in water, those that adhere through non specific, non-covalent interactions and those that bind to specific receptor sites on the cell surface (Park & Robinson, 1984).

An ideal polymer for a bioadhesive delivery system should have the following characteristics (Jimenez-Castellanos et al, 1993; Langer & Peppas, 1981);

- The polymer and its degradation products should be non-toxic and non absorbable from the gastro-intestinal tract.
- It should be non-irritant to the mucous membrane.
- It should preferably form a strong non-covalent bond with the biological substrate.
- It should adhere quickly to the tissue and possess some site specificity.
- It should allow easy incorporation of the drug and allow release from the formulation.
- The polymer must not decompose on storage or during the shelf life of the dosage form.
- The cost of the polymer should be low enough for the formulated dosage form to remain competitive.

The presence of charged groups and/or hydrophilic functionalities such as carboxyl, hydroxyl, amine or sulfate groups are beneficial to good bioadhesion (Park & Robinson, 1984; Smart & Kellaway, 1982). These groups aid interactions via electrostatic or hydrogen bonding. Anionic polymers show greater bioadhesive potential than cationic molecules and they also show a greater degree of interpenetration with mucosal substrates (Mortazavi & Smart, 1994). The flexibility of a polymeric chain also has an effect on the degree of interaction. Flexible chains are able to orientate to enhance the number of binding sites available. The degree of hydration plays an important role in the adhesion of a polymeric moiety (Gu et al, 1988). Too much water may lead to a slippery interface and minimal adhesion, on the other hand too little water may prevent movement of the polymer and interpenetration will be hindered. Studies have shown that the degree of bioadhesion decreases with increasing water content within the formulation (Nguyenxuan et al, 1996; Woolfson et al, 1995). The effect of molecular weight on the adhesion of a polymer is controversial. Early studies reported that the adhesive strength increased as the molecular weight of the polymer increased up to approximately one hundred thousand, after which there was little effect (Huntsberger, 1967). Linear polymers have been reported to have an increasing adhesive strength up to a molecular weight value of four million (Chen, 1970). Coiled polymers do not show such obvious trends in the relationship between adhesive strength and molecular weight. This is probably due to shielding of interaction sites and intra-molecular binding rather than intermolecular interactions.

It is very difficult to compare the relative merits of different polymers as bioadhesive agents as no test is all encompassing and gives a result that is applicable to all situations. Some studies have been performed to rank bioadhesive polymers in order of their bioadhesive strength. Wong et al (1999a) used a chicken cheek pouch to simulate buccal delivery and ranked the following polymers in order of decreasing bioadhesive strength Carbopol > gelatin > sodium carboxymethyl-cellulose >
hydroxypropylmethylcellulose > alginic acid = chitosan. Although a second study by Wong et al (1999b), showed the order to be sodium carboxymethyl-cellulose > xanthan gum > Carbopol. A further study used an in vivo periodontal model to show that the bioadhesive potential was ranked; xanthan gum > poly (ethylene oxide) > chitosan (Needleman et al, 1997). The polymers were ranked poly(acrylic acid) > sodium carboxymethylcellulose > hydroxypropylcellulose by Cvetkovic et al (1997) using an in vitro modified intestinal perfusion technique. These conflicting results show that there is discrepancy in the literature reports of the bioadhesive potential of polymers. The results show that according to the method used the bioadhesive potential can vary a great deal.

Physiological variables may affect the strength of the bioadhesive interaction and also the properties required to induce a strong interaction. The turnover time of mucus or the flow of saliva may mean that the time period for which the bioadhesive can remain at the site of action is shorter than desired. The nature of the biological substrate may be altered during disease conditions, if the bioadhesive is to be used in the disease state the bioadhesive properties need to be assessed under these conditions. All these physiological phenomena must be considered in any experimental design.

1.3.4 Bioadhesive dosage forms

The gastro-intestinal tract as a whole is an attractive route for bioadhesive drug delivery as the mucosal surface is moist and mainly served by a rich blood supply. The disadvantages of targeting the gastrointestinal tract include the harsh physiological conditions including the many secretions and forces that act throughout the tract. Bioadhesive systems have been developed in order to increase local delivery (Smart, 1993).

1.3.4.1 Biohesion within the oral cavity

Bioadhesive dosage forms targeted at many sites within the body are available commercially. The oral cavity is one of the most accessible areas that is ideally
suited to bioadhesive drug delivery. Many different formulations are available to treat diseases present in the oral cavity including liquids (mouthwashes), semi-solids (gels or pastes) and solids (lozenges, patches or tablets) (Washington & Wilson, 1989). However, these systems have encountered difficulties both due to the flushing action of saliva and the forces involved in chewing and swallowing. The buccal cavity is an area within the oral cavity that is less exposed both to salivary flow and the forces involved in eating. For this reason the buccal cavity has been extensively investigated as a site of bioadhesion as it offers little resistance to drug absorption and also as a means of avoiding first pass metabolism (Leung & Robinson, 1992). Other advantages of the buccal cavity include the safety aspect whereby in cases of toxicity the dosage form can be simply and quickly removed from this site. These phenomena make the buccal route particularly attractive for peptide delivery. Current formulations used in the buccal cavity include Orabase® and Zilactin®, used to treat mouth ulceration locally. Other drugs are also available for systemic action as buccal delivery systems including prochlorperazine maleate and captopril.

1.3.4.2 Bioadhesives within the œsophagus

Very little work has been performed investigating the œsophagus as a section of the gastro-intestinal tract involved with bioadhesive systems. There are, however, disorders of the œsophagus including GORD and œsophageal cancer, where prolonged local drug retention is required (Orlando, 1991b; Ito et al, 1990). Delivery to the œsophagus may provide a means by which first pass metabolism could be avoided. Disadvantages of the œsophagus as a site of drug delivery include the small surface area of 157 cm² (Washington, 1991) and the relatively low permeability of the stratified squamous epithelium. Bioadhesive formulations targeted at the œsophagus have focused on flowable liquids containing mucoadhesive polymers (Dobrozsi et al, 1999). As force of detachment tests are unsuitable to assess the adhesive strength of liquid formulations, other methods have been developed to assess the mucoadhesion of formulations targeted at the œsophagus. Iooss et al (1995) investigated the retention and drug release of δ-aminolevulinic acid in a continuous flow column adhesion cell and found that polycarbophil showed the best retention. δ-aminolevulinic, when applied directly to
the œsophageal mucosa may be used to treat Barrett's œsophagus via photodynamic therapy. Ito et al (1990) used magentic granules to deliver anti-cancer agents to the œsophagus, whereby magnetism was used to target the granules during administration and bioadhesive polymers were employed to aid retention upon removal of the magnet. This method used an agar tube as an œsophageal model although in vivo retention has been shown in rabbits (Nagano et al, 1997).

Contrary to other sites within the GI tract polymeric adhesion systems take second place to a complex of aluminium hydroxide and sucrose octasulfate (sucralfate). Sucralfate is indicated in the treatment of ulcers (Ishimori, 1995). Sucralfate adheres to œsophageal tissue in a different manner to polymers thus the theories of bioadhesion discussed do not apply to the adhesion of sucralfate. The mechanism of retention of sucralfate is thought to be due to acid-induced chemical transformation of the drug from an insoluble powder to a swollen adhesive paste at the site of ulceration (Nagashima & Yoshida, 1979). Dobrozsi et al (1999) used an ex vivo technique to assess the retention of sucralfate suspensions on œsophageal tissue. The study used rat œsophageal mucosa to compare the retention of Carbopol and three commercial sucralfate suspensions. The results showed that a sucralfate suspension prepared in gel form possessed greater retention than other systems. These results were consistent with human œsophageal retention data indicating that this model has potential in screening œsophageal adhesive agents.

Other studies performed on adhesion within the œsophagus have included reports of tablets lodging (Al-Dujaili et al, 1986). This phenomenon can have a damaging impact on the œsophageal mucosa and can even lead to ulceration with certain drugs.

1.3.4.3 Bioadhesives within the stomach

The gastric mucosa has been extensively investigated as a target for bioadhesive drug delivery systems. A bioadhesive formulation would increase the residence time within the stomach and enhance the absorption profile of a drug. However, gastric motility and muscular contractions may dislodge any such device. The density must also be considered in the development of a bioadhesive formulation targeted at the stomach as a formulation with a density lower than that of the stomach contents may
be able to float on the gastric contents then form an interaction with the exposed gastric mucosa. A very dense tablet would integrate with the gastric contents and be shielded from the mucosal surface.

Many studies have been performed to assess the mucoadhesive strength of various polymers targeted at the gastric region. Park & Robinson (1984) showed that polycarbophil demonstrated the best adhesive properties in both the stomach and the small intestine. Other work performed (Rao & Buri, 1989) showed that polycarbophil and sodium carboxymethylcellulose demonstrated the best adhesive strength. All studies performed have been ex vivo. As yet, there are no commercial adhesive formulations targeted to the stomach.

1.3.4.4 Bioadhesive delivery to the nasal cavity

The nasal cavity possesses many advantages as a site for drug delivery including a large surface area that is highly vascularised. Absorption from this site also avoids first pass metabolism. A disadvantage associated with delivery within the nasal cavity is the rapid mucociliary clearance mechanisms.

Drugs can be administered to the nose for topical or systemic action, avoiding first pass metabolism. The rich vascularity of the nasal membranes and the ease of intranasal administration offers an ideal alternative route of administration. Bioadhesive microspheres were developed for nasal administration (Illum et al, 1987). The half-life of clearance was found to be in the order of 240 minutes, compared to 15 min for liquid and powder control formulations.

1.3.4.5 Bioadhesives for ophthalmic application

In ophthalmic therapy one of the major problems is to produce and maintain an adequate concentration of the drug at the site of action for a prolonged period of time. Tear production and mucin turnover make this a difficult organ to target and standard formulations require administration at least four times daily (Robinson, 1989). Another important aspect of bioadhesive systems targeted to the eye is that there is no interference with vision. Unlu et al (1991) investigated acrylic polymers
Chapter One: Introduction

and their applicability as bioadhesive delivery systems targeting the eye. They are non-irritant, well tolerated and present no interference to visibility in situ. A review is available that details mucoadhesive ocular drug delivery systems (Robinson, 1990).

1.3.4.6 Vaginal application of bioadhesive formulations

Vaginal delivery is often considered for drugs that are susceptible to gastro-intestinal degradation or extensive hepatic metabolism. Bioadhesive systems can also be used as moisturisers in cases of vaginal dryness. A bioadhesive system designed for vaginal delivery would increase the residence time and a greater bioavailability may be achieved. Antibiotics, antifungal agents and systemically acting contraceptives are commonly administered to the vaginal cavity; a bioadhesive delivery system could increase the bioavailability of such systems. Further information about vaginal drug delivery systems can be found in a review by Robinson (1992).

1.3.4.7 Bioadhesive agents targeted for rectal administration

The rectum is only 10 cm long with a relatively small surface area (Leung & Robinson, 1992), however, due to the easy accessibility of this region and the avoidance of first pass metabolism in areas close to the anus, the rectum is a good potential site for bioadhesive agents. Conventional suppositories can migrate from the lower rectal area where the blood drains directly into the systemic circulation to the upper region where the blood supply is subjected to liver metabolism. The use of a bioadhesive formulation would reduce the tendency of migration. Hydrogels have shown potential in rectal drug delivery although bioadhesives have not been extensively employed in this region. Table 1.3 lists some bioadhesive drug delivery systems that are commercially available.
**Table 1.3.** Comparison of some commercially available mucoadhesive preparations (Kamath & Park, 1994). (HPMC - hydroxypropylmethylcellulose; NaCMC - sodium carboxymethylcellulose)

<table>
<thead>
<tr>
<th>Name and Form</th>
<th>Drug</th>
<th>Mucoadhesive agent</th>
<th>Target Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aftach tablet</td>
<td>Triamcinolone acetonide</td>
<td>HPMC, Carbopol</td>
<td>Oral cavity</td>
</tr>
<tr>
<td>Buccastem tablet</td>
<td>Prochlorperazine maleate</td>
<td>Ceratonia and Xanthan gums</td>
<td>Buccal</td>
</tr>
<tr>
<td>Salcoat tablet</td>
<td>Beclomethasone dipropionate</td>
<td>HPMC</td>
<td>Oral Cavity</td>
</tr>
<tr>
<td>Solcoseryl paste</td>
<td></td>
<td></td>
<td>Gingival</td>
</tr>
<tr>
<td>Orabase gel</td>
<td></td>
<td>NaCMC, Pectin and Gelatin in Polyethylene-mineral oil base</td>
<td>Oral Cavity</td>
</tr>
<tr>
<td>Rhinocort powder spray</td>
<td>Beclomethasone dipropionate</td>
<td>Hydroxylpropylcellulose</td>
<td>Nasal</td>
</tr>
<tr>
<td>Replens gel</td>
<td>Aluminium hydroxide</td>
<td>Polyacrylic acid</td>
<td>Vaginal</td>
</tr>
<tr>
<td>Sucralfate</td>
<td></td>
<td>Sucrose octasulfate</td>
<td>Gastrointestinal ulcers</td>
</tr>
</tbody>
</table>

Having examined the phenomena surrounding bioadhesion and briefly discussed the sites of adhesion that have been investigated it is interesting to link the work performed so far. The final part of the introductory chapter will focus on the physical and chemical properties of alginate and the use of alginate in bioadhesive drug delivery systems.

### 1.4 ALGINATES

Alginites were discovered and named in the latter part of the nineteenth century by the British chemist E. C. Stanford (McNeely & Petitt, 1973). Stanford extracted these naturally occurring polysaccharides from brown seaweed and noted their thickening, gelling and film forming properties.
Alginate molecules are located in the intracellular spaces of seaweed. They provide the plant with both the flexibility and strength to withstand the seabed environment. Alginate is found in all species of brown algae. Commercial alginate is sourced from many different species including *Laminaria hyperborea*, *Lessonia* species, *Laminaria digitaria* and *Durvillaea* species. Certain bacteria also produce alginate as an exopolysaccharide. Both *Pseudomonas* and *Azotobacter* species have been investigated (Rehm & Valla, 1997).

1.4.1 Alginate chemistry

The alginate polysaccharide chain is built up from two monomeric sugar units α-L-guluronic acid (G) and β-D-mannuronic acid (M). The structures of these uronates are shown in Figure 1.8.

The proportion and distribution of these monomers and their relative sequencing determines the chemical and physical properties of the alginate molecule. In its natural environment, the composition of the chain varies according to the species and source of the alginate. In nature, the alginate within the stem is required to be rigid and has a different composition to that in the leaves. Variations in the monomeric make-up are also found according to season.

![Structure of guluronate and mannuronate](image)

**Figure 1.8.** The structure of guluronate and mannuronate

Alginates exist as block copolymers; the monomer units are arranged in regions, both polyguluronate and polymannuronate blocks occur as well as sequences of an alternating character. Thus is can be said that the distribution of these monomer
units along the alginate chain is neither random nor does it follow any statistical model (Smidsrød & Draget, 1997). Figure 1.9 shows a schematic representation of an alginate chain.

![Schematic alginate chain sequence](image)

**Figure 1.9.** Schematic alginate chain sequence

X-ray diffraction studies on the polymannuronate sequences showed that the units linked together in a $^4\text{C}_1$ diaxial linkage (Atkins et al, 1973a) and polyguluronate blocks linked in a $^1\text{C}_4$ diequatorial conformation (Atkins et al, 1973b). These conformations are the most energetically favourable structures for each of the monomer chains. Figure 1.10 shows the bonding between monomer units within an alginate chain.

![Chemical structure of alginate](image)

**Figure 1.10.** Chemical structure of alginate

It is the bulky carboxylic acid group that determines the bond orientation. In G blocks this group leads to a diaxial linkage around which rotation is hindered, the diequatorial glycosidic bond within M blocks is less hindered allowing M blocks more flexibility. Hydrogen bonding between adjacent molecules also contributes to the freedom of rotation around the glycosidic linkage. Figure 1.11 shows the weak
hydrogen bonds present between adjacent mannuronate residues within an alginate chain.

![Hydrogen bonding within polymannuronate sequences](image1)

**Figure 1.11.** Hydrogen bonding within polymannuronate sequences

The dotted line represents a hydrogen interaction that can occur between adjacent mannuronate residues. These hydrogen bonds act to strengthen the molecule; they can act between adjacent residues within a chain or as linkers between mannuronate residues on adjacent alginate chains (Atkins et al, 1973a).

The diaxial linkage present in polyguluronate blocks leads to a buckled ribbon structure with very limited flexibility (Atkins et al, 1973b). A strong hydrogen bond exists between the carboxyl group on one residue and a hydroxyl group on the adjacent residue; this is shown in Figure 1.12.

![Hydrogen bonding within polyguluronate sequences](image2)

**Figure 1.12.** Hydrogen bonding within polyguluronate sequences

The dotted line represents a strong hydrogen bond that retains the diaxial linkage present in G blocks. The interaction here is stronger than in the adjacent mannuronate residues as it is an acid group that is linking rather than a hydroxyl group.
The difference in the inter-unit binding between the two monomer units has implications for the properties of the alginate chain. G-rich areas of an alginate chain are rigid and brittle whereas the M-rich blocks are flexible and have elastic properties (Smidsrød & Draget, 1996). In nature the leaves of seaweed plants have a greater portion of M residues than G, while the opposite is true in the stem. This difference is found due to the difference in the nature of these residues, the G residues are stronger and more rigid thus better suited to the supportive role of the stem, whereas flexibility is desirable for the leaves (Pronova Biopolymer Handbook).

1.4.2 Alginate stability

Dry, powdered, pure sodium alginate may have a shelf life of months provided it is stored in a dry, cool place without exposure to sunlight. In the freezer sodium alginate may keep for years with no significant reduction in molecular mass. Dried alginic acid has a very limited shelf life due to an intramolecular acid catalysed hydrolysis reaction (Christensen et al, 1996).

1.4.3 Alginate gelation

The strength, porosity, size, shape and homogeneity of an alginate gel can be varied by both the gelling mechanism and the alginate composition, thus enabling a variety of gels to be manufactured and tailored to meet specific requirements. Alginates form gels in two different ways; at low pH values and in the presence of free cations of an appropriate size (Moe, 1993).

Mannuronic acid and guluronic acid have pK$_a$ values of 3.38 and 3.65 respectively (King, 1983). At pH values below the pK$_a$ of the alginate molecule a gel is able to form. Protonation of the alginate molecule reduces the electrostatic repulsion from between carboxylate anions, this leads to the formation of hydrogen bonds both within and between the alginate molecules resulting in adjacent chains interacting to form a gel network.
Alginate composition has an effect on the strength of acid gels, long stretches of G-residues give the strongest gels, and polymannuronate sequences also promote gelation however, alternating sequences appear to perturb gel formation (Moe, 1993). The greater relative gel strengths associated with homopolymeric block structures suggests that co-operative processes are involved in the gel formation.

Diaxially bound polyguluronate sequences provide binding sites for divalent cations which are bound selectively in the following range; Mg > Ni > Ca > Sr > Ba > Zn > Cd > Cu > Pb (Grant et al, 1973). Ionically cross-linked alginate gels are used commercially and most work using calcium ions as the cross-linker. Figure 1.13 shows a representation of a calcium ion binding into the site between two guluronate residues.

![Figure 1.13. Schematic representation of the calcium-alginate interaction](image)

It was originally proposed (Grant et al, 1973) that a second alginate chain sandwiched the cross-linking agent between two chains to form a gel. However, there has been a recent debate about this theory and it is now thought that the cation may cross link more than two alginate chains at high alginate concentrations (Smidsrød, 1999).

In general the elastic modulus of an alginate gel depends on the number and strength of cross links and the length and stiffness of the chains between the linkers. The modulus of calcium alginate gels depends strongly on alginate composition; G-rich alginates form strong brittle gels whereas M-rich alginates form softer, less brittle gels (Moe, 1993). The diaxial linkage present in polyguluronate chains provides sites for the cation to link alginate chains, thus more cross-linking agents are present to form a more cohesive gel.
1.4.4 Uses of alginates

Alginates are used for a variety of functions in many different industries. Table 1.4 lists the industries that use alginate and the function for which it is used.

Table 1.4. Applications of alginates (Onsoyen, 1996)

<table>
<thead>
<tr>
<th>Application</th>
<th>Function of alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Textile printing</td>
<td>Fixation, colour yield and brightness, ensuring even printing</td>
</tr>
<tr>
<td>Dental materials</td>
<td>Gel formers in dental impression materials</td>
</tr>
<tr>
<td>Paper treatments</td>
<td>Coating agents</td>
</tr>
<tr>
<td>Food industry</td>
<td>Stabilisers in emulsions e.g. salad dressings</td>
</tr>
<tr>
<td></td>
<td>Foam stabiliser in beer</td>
</tr>
<tr>
<td></td>
<td>Gelling agent in desserts</td>
</tr>
<tr>
<td>Pharmaceutical and biotechnology</td>
<td>Heartburn remedies</td>
</tr>
<tr>
<td></td>
<td>Wound treatment</td>
</tr>
<tr>
<td></td>
<td>Cosmetics</td>
</tr>
<tr>
<td></td>
<td>Drug delivery systems</td>
</tr>
</tbody>
</table>

One pharmaceutical use of alginates is in the preparation of gel beads that are used to encapsulate both complex macromolecules and cells. This system has been developed and has possible implications in the potential use of alginate-encapsulated pancreatic islet cells for the treatment of type I diabetes (Lim & Sun, 1980), and also as a controlled drug release formulation (Rajaonarivony et al, 1993). A second pharmaceutical use of alginates involves the covalent attachment of drugs and drug carriers to alginate chains (Morgan et al, 1995). Covalent attachment of such molecules to macromolecular carriers has the potential of developing sophisticated drug delivery systems with improved bioavailability and pharmacokinetics (Duncan, 1992).

Alginates have recently found applications in the treatment of wounds. Fibracol®, Kaltocarb®, Sorbsan® and Tegagen® are all dressings that contain alginate as an ingredient. Alginate dressings maintain a physiologically moist environment that
promotes healing and the formation of granulation tissue. Such a dressing can be rinsed away with saline irrigation so removal of the dressing does not interfere with healing granulation tissue (Gilchrist & Martin, 1983).

Alginate is an active component of Gaviscon Liquid® (Reckitt & Colman Products) which is used to treat gastro-oesophageal reflux disorder (GORD). The Gaviscon Liquid® formulation works by a physical mechanism that does not rely on absorption into the systemic circulation. The alginate preparation reacts with gastric acid to form a strong viscous gel or raft of near neutral pH that floats on the gastric contents. The advantage of this type of formulation over a simple antacid is that the preparation remains in the upper part of the stomach for a longer period of time (Washington et al, 1987). The raft impedes reflux of gastric contents into the oesophagus; in more severe cases the raft itself can be refluxed (Malmud et al, 1979) preventing contact between gastric acid and the oesophageal mucosa. As this project explores the retention of a liquid alginate formulation on oesophageal tissue it is pertinent to investigate the bioadhesive properties of sodium alginate and the instances when alginate has been used in a bioadhesive drug delivery system.

1.4.4.1 Alginates as bioadhesive agents

Alginates have been reported to be poor bioadhesives by Charrueas et al (1993) although other reports (Chen & Cyr, 1970; Smart et al, 1984) suggest that alginates are excellent bioadhesive agents. This discrepancy is probably due to the lack of a universal test, as stated previously. Alginates have been used in several bioadhesive formulations within the pharmaceutical industry. Alginate has been investigated (Witschi & Mrsny, 1999) as a potential bioadhesive system targeting the nasal cavity as a means of increasing the bioavailability of protein therapeutics. This study showed that the alginate released the protein efficiently but was not as adhesive as either chitosan or carbopol within the nasal cavity. Ocular delivery of alginate-based bioadhesives has also been investigated as a delivery system for pilocarpine (Cohen et al, 1997).
The oral cavity has been investigated as the site of adhesion of alginate based bioadhesive tablets. Alginate in combination with chitosan has shown (Miyazaki et al, 1994) strength of adhesion values to be equivalent to formulations in clinical use within the oral cavity. Alginate - sodium carboxymethylcellulose tablets have been investigated (Efentakis et al, 1998) as a potential delivery system for tetracycline within the oral cavity. Alginate beads or microcapsules have been widely investigated as a bioadhesive drug delivery system targeting the gastric mucosa (Takahashi et al, 1990; Gaserod et al, 1998). These beads have been investigated as pure alginate beads and also beads coated with second polysaccharides including chitosan (Lee et al, 1997). A liquid alginate suppository formulation has been investigated as an acetaminophen delivery system targeting the anus (Kim et al, 1998).

Alginates have been investigated as drug delivery systems to many sites within the body in many different formulations. Investigation of the adhesion of a liquid alginate formulation on oesophageal tissue was performed by Banning (1999). The aims of this project are to characterise this adhesive interaction. This introduction has covered the target organ, the mechanisms involved in the bioadhesive process and also the relevant aspects of alginate chemistry in order to provide an overview of the project background.

1.5 AIMS AND OBJECTIVES OF THE STUDY

The aim of this project was to characterise the adhesion of alginate solutions on oesophageal tissue. The rationale behind this work was to enhance the protective defence mechanisms within the oesophagus to aid against damage caused by gastric reflux. An additional adhesive layer present on oesophageal tissue may limit the damage caused to this organ by the reflux of the acidic gastric contents. The ability of such an adhesive layer to support the incorporation of model drug particles was also investigated.
1.5.1 Objectives

- To investigate oesophageal tissue as a substrate for adhesive systems. The surface properties were characterised in order to optimise delivery to this site.

- To develop and validate a suitable method to assess bioadhesive properties of a liquid system. The extent and duration of retention were quantified using such techniques.

- To determine how the surface properties of oesophageal tissue affect the retention of an alginate dose as assessed using the method developed above.

- To assess how the alginate physico-chemical properties affect the retention of a set alginate dose on oesophageal tissue. Again this was assessed using the methods developed above.

- To determine the feasibility of the adhered alginate layer in supporting model drug particles. The retention of model drug particles of a variety of sizes and charges was assessed using the model as developed.
2.1 INTRODUCTION

A number of different techniques were used within this project in order to characterise the adhesion of alginate solutions to porcine esophageal tissue. This chapter describes the basic materials and techniques used throughout the project. Further details and background information on the specific instrumentation and techniques used can be found in the designated experimental chapters.

Certain materials that were common to several procedures within the project are also described in this chapter. All chemicals used were stored according to the supplier’s instructions and any further analysis or purification of a material is detailed within this section. Statistical analyses performed on the results obtained are also explained within this chapter.

2.2 SODIUM ALGINATE

Sodium alginate powder, as supplied by FMC Biopolymer (Norway), was stored under the suggested conditions of a cool place (at 4 – 7 °C) removed from sunlight exposure. Under these conditions sodium alginate is reported to be stable for several months (Smidsrød & Draget, 1996). A range of sodium alginates was investigated. The data sheet, supplied with each alginate contained information on the dry material content, calcium content, sodium content, molecular weight and monomeric make up of the alginate chain. These details are listed in Table 2.1. The viscosity and pH value of a 1 % w/v aqueous solution of the alginate were also given. However, 2 % w/v solutions were utilised within the study. Thus the viscosity of such solutions were measured and the data is also presented in Table 2.1. The viscosity values of the 2 % w/v solutions were calculated within the laboratory according to the following method. A TA Instruments (UK), AR1000N controlled stress rheometer
was used to determine the viscosity of these solutions. All viscosity measurements were taken at 37 °C. No further analysis was performed on the alginates as previous work has shown the manufacturers analysis to be sufficient (Banning, 1999). The chemical and physical properties of the alginates used are shown in Table 2.1.

Table 2.1. Comparison of the properties of the range of alginates used in the study. All the data listed was supplied by the manufacturer with the exception of the viscosity values that were calculated as described previously

<table>
<thead>
<tr>
<th>Alginate</th>
<th>Batch Number</th>
<th>Dry Weight (%)</th>
<th>Molecular Weight (KDa)</th>
<th>Fraction of G units</th>
<th>Viscosity of a 2% Solution (Pa s) (shear rate = 10 s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF120</td>
<td>645209</td>
<td>90.6</td>
<td>240</td>
<td>0.44</td>
<td>0.51</td>
</tr>
<tr>
<td>SF120</td>
<td>499231</td>
<td>91.0</td>
<td>220</td>
<td>0.69</td>
<td>0.43</td>
</tr>
<tr>
<td>H120L</td>
<td>645209</td>
<td>86.4</td>
<td>416</td>
<td>0.46</td>
<td>4.12</td>
</tr>
<tr>
<td>SF200</td>
<td>511322</td>
<td>90.4</td>
<td>387</td>
<td>0.69</td>
<td>2.93</td>
</tr>
<tr>
<td>LF10L</td>
<td>840017</td>
<td>89.2</td>
<td>75</td>
<td>0.45</td>
<td>0.02</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>599345</td>
<td>85.3</td>
<td>40</td>
<td>0.64</td>
<td>7.49 x10^{-3}</td>
</tr>
</tbody>
</table>

Unless otherwise stated, sodium alginate LF120 was used in the majority of the work. The other alginates were used to compare how the nature of the physico-chemical properties of the alginate affected the interaction. LF120 was selected as its physico-chemical properties are mid-range from the alginates available. A 2 % w/v concentration was chosen, as its viscosity at a shear rate of 10 s^{-1} is similar to that of Gaviscon Liquid®, a commercially available alginate containing formulation used in the treatment of gastro-oesophageal reflux disease. At a shear rate values of 10 s^{-1} and a temperature of 37 °C the viscosity of Gaviscon Liquid® was found to be 0.46 (± 0.02) Pa.s compared to 0.51 (± 0.02) for 2 % LF120. These data were collected according to the method described previously.

The dry weight of sodium alginate powder was used for calculating the required mass to produce the set concentration when preparing all solutions. These solutions were prepared using a Heidolph RZR 50 rotary mixer at a speed of 2000 rpm in
conjunction with a 20 mm diameter, three-blade propeller. Deionised water was stirred at a speed of approximately 1800 rpm to induce a vortex and sodium alginate powder was added slowly into the middle of this vortex. The propeller speed was then increased to 2000 rpm and left to mix for 20 minutes until all the alginate powder was dissolved. Any alginate present in clumps that had not been dissolved were broken up and stirred into the alginate solution using a spatula. This solution was mixed again using the rotary mixer in order to produce a homogenous product.

Sodium alginate solutions used in both the retention study and also within the microscopy investigations were fluorescently labelled, either by covalent attachment of a fluorescent molecule or by preparation in a solution of disodium fluorescein. The procedures involved in these processes are outlined below.

### 2.2.1 Covalent attachment of a fluorescent label to sodium alginate

Fluoresceinamine (Isomer I) was purchased from Sigma, UK. This has been used previously as a fluorescent label for covalent binding to sodium alginate using a method described by Blonk et al (1995). The principle of this method involves attaching the amine group of the dye with a small fraction of the carboxylic acid groups present in the alginic acid chain. Dioxane was present to help solubilise the dye. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) acted as a dehydrating catalyst to remove water and thus enable the amide bond to form. The procedure and mechanisms involved in the labelling process are outlined below. Table 2.2 lists the chemicals used in the procedure.
Table 2.2. The chemicals used in the fluorescent labelling of sodium alginate with fluoresceinamine

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
<th>Batch number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium alginate (LF120)</td>
<td>FMC, Biopolymer</td>
<td>499231</td>
</tr>
<tr>
<td>Fluoresceinamine (isomer I)</td>
<td>Acros</td>
<td>A010677901</td>
</tr>
<tr>
<td>EDC</td>
<td>Lancaster</td>
<td>10025464</td>
</tr>
<tr>
<td>Dioxane</td>
<td>BDH</td>
<td>J429314</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>M&amp;B</td>
<td>ML0511</td>
</tr>
<tr>
<td>Acetone</td>
<td>Lab-Scan</td>
<td>4272/9</td>
</tr>
<tr>
<td>Diphosphoropentoxide</td>
<td>BDH</td>
<td>ZU266178</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>BDH</td>
<td>B301548</td>
</tr>
</tbody>
</table>

2.2.1.1 Covalent labelling procedure

5 g of sodium alginate (LF120) was dissolved in 400 ml deionised water. Concentrated sulfuric acid was added drop-wise in order to lower the pH and thus precipitate the alginic acid in its gel form. The system was centrifuged (Beckman J2-21 instrument with a JA14 rotor) twice with water and twice with acetone at 15000 rpm for 30 minutes each time. This was necessary to separate the alginic acid pellet from the soluble ions and other impurities that may have been present within the system. The pellet was removed and dried in a dessicator above diphosphoropentoxide at room temperature. The alginic acid pellet produced was then crushed and ground using a pestle and mortar. It was subsequently added to 50 ml of deionised water. This was stirred for at least 10 minutes in order to solubilise as much of the alginic acid as possible. 1 g EDC was then added in order to begin the initial part of the dehydration reaction. 50 mg fluoresceinamine was dissolved in 30 ml 1,4-dioxane. This second solution was added slowly to the alginate/EDC solution whilst stirring. Dissolving the dye in the dioxane aided the dispersity of the fluorescent label producing a homogenously labelled alginate solution. This solution was stirred overnight. Due to the length of this procedure, the beaker containing the solution was wrapped in foil to prevent light degradation of the fluorescent
component. The reaction solution was then neutralised slowly using concentrated sodium hydroxide solution and the pH monitored throughout this process (Hanna Instruments 8520 pH meter; calibrated at both pH 4 and at pH 7). The amide bond is very susceptible to alkali hydrolysis thus it is better to remain on the acidic side of neutral to produce a stable product. This pH 7, neutral solution was then exhaustively dialysed against deionised water to remove any free dye or other small molecular weight impurities. Once the dialysis was complete the labelled alginate solution was freeze dried (Edwards Micro Modulyo with an Edwards RV3 pump) and stored in the same manner as ordinary sodium alginate. The mechanism of this reaction is shown in Figure 2.1.

\[
\text{Alginate} + \text{EDC} \rightarrow \text{Intermediate} + \text{Fluoresceinamine} \rightarrow \text{Labelled alginate}
\]

**Figure 2.1.** The mechanism involved in the preparation of fluorescently labelled alginate chain
The resulting compound was an alginate chain with fluorescent moieties distributed along the molecule. An estimation of the percentage substitution can be calculated assuming 100% substitution. The molar ratio of the dye to the alginic acid monomer was equal to $5.5 \times 10^3$. This value can thus be assumed to be the percentage substitution of the dye to the alginate. The excitation and emission absorption frequencies of an aqueous solution were found to be 489 and 515 nm respectively. The viscosity of a 2% solution of the labelled alginate was found to be 0.75 (± 0.03) Pa.s at a shear rate of 10 s$^{-1}$ and 37 °C as compared to 0.51 (± 0.02) Pa.s for the unlabelled 2% LF120. This higher viscosity suggests that very little degradation of the alginate chain occurred during the labelling process and that the process may even aid the formation of a strong polymeric network.

Gel permeation chromatography was performed to assess the purity of the resulting labelled alginate. This method ascertains the size distribution of entities present within a solution and separates them according to molecular weight. A gel filtration chromatography column contains a stationary phase consisting of porous beads with a well-defined range of pore sizes. Molecules that are small with respect to pore size can fit inside the pores within the beads have access to the internal mobile phase as well as the external mobile phase between beads and therefore, elute last in a gel filtration separation. Molecules that are large with respect to pore size are excluded and therefore elute first. As the molecular weight of the dye is very low compared to the labelled alginate chain, these two entities should be separated and will be seen as two peaks using gel filtration chromatography. This example could be visualised within the column due to the fluorescent nature of the dye. The two entities, labelled alginate and free dye, are both visible due to their fluorescence. Therefore a separation of the two materials would result in the visualisation of two separate, distinct bands of colour moving at different rates through the column. This phenomenon was not observed and the fractions collected exhibited unimodal distribution, which is indicative of the presence of only one fluorescent material. This is shown schematically in Figure 2.2.
2.2.2 Fluorescein-alginate solution

Figure 2.2. Schematic representation of the eluted material from the gel filtration column

The result shown in Figure 2.2 was obtained by running 1 ml of a 1 mg/ml solution of the labelled alginate down a calibrated Sephadex G-25 pre-packed column. Only one peak was observed indicating that very little or no unbound dye was present in the solution. Although this technique shows that the labelled alginate is the predominant material within the 1 mg/ml solution it does not quantify the extent or homogeneity of substitution by the fluorescent dye.

A second “labelling” technique was used whereby the alginate powder was added to a solution of disodium fluorescein. This second fluorescent alginate solution was utilised as it provided a very simple and repeatable procedure. The covalent labelling technique was time consuming and the repeatability and extent of substitution were not quantified. However, the covalent label provided a more definite marker to represent the location of the alginate moiety within a given sample.
This second fluorescent solution cannot really be described as labelled alginate as there is no interaction between the alginate and dye molecules. Disodium fluorescein is a water-soluble fluorescent dye. At high concentrations it has a yellow-red colour with intense yellow-green fluorescence detectable at 0.02 parts per million. The method used in the preparation of this labelled alginate solution is outlined below. The excitation and emission wavelengths of the prepared aqueous alginate solution "labelled" with this dye were found to be 490 nm and 515 nm respectively. The fluorescence disappears in acidic conditions but will reappear in a neutral or alkaline solution.

Figure 2.3 shows the chemical structure of disodium fluorescein. The chemical formula is C_{20}H_{10}Na_{2}O_{5} and the molecular weight is 376.28. This dye is also termed "acid yellow". The disodium fluorescein salt was obtained from Avocado research chemicals Ltd., Lancashire (batch number A2850A).

![Figure 2.3. The chemical structure of disodium fluorescein](image)

### 2.2.2.1 Preparation of a solution of alginate in sodium fluorescein

A 2 % w/v alginate solution was prepared in a 0.001 % fluorescein solution. This sodium fluorescein solution was prepared by initially weighing 0.1 g of disodium fluorescein salt and making this up to 100 ml with deionised water in a volumetric flask. 1 ml of the above solution was diluted to 100 ml with deionised water to produce a 0.001 % disodium fluorescein solution. The relevant mass of sodium
alginate was added to this solution and the alginate solution was prepared as described previously. This solution relies on the fact that the alginate and fluorescein remain associated within the aqueous solution. Although this method is not ideal, it has advantages compared to the covalent labelling technique. The alginate-sodium fluorescein solution is readily prepared and the batch-to-batch variation is minimised. The disadvantages include the possibility of the fluorescent marker dissociating from the alginate thus leading to discrepancies in the results. The two labelling techniques were compared in the relevant experimental chapters.

2.3 PREPARATION OF THE OESOPHAGEAL TISSUE

Porcine oesophageal tissue was used as a substrate. Oesophageal tissue used was prepared from fresh porcine oesophagi collected from the abattoir on the day of sacrifice. The tissue was collected and kept at –4 °C for transportation from the abattoir to the laboratory. Once received in the laboratory, the oesophagi were kept on ice prior to removal of the outer muscular layers. This procedure was performed by cutting through the muscle layers to expose the inner epithelial tube. Once this tube was visible the muscle layers were carefully peeled away to minimise any damage to the epithelial tube. The tissue was then cut into 10 cm lengths, flash frozen in liquid nitrogen and then stored at –20 °C until required. The day before the tissue was required it was removed from the freezer, allowed to thaw overnight in the fridge and stored in the fridge until required. This method of preparation has been shown to retain the histological integrity of the tissue (Young & Smart, 1998).

2.3.1 Preparation of the oesophageal scrapings

The unstirred water layer resident on oesophageal tissue was removed from the tissue surface in the form of oesophageal scrapings. These scrapings were taken from the porcine oesophageal tissue prepared as described above. The scrapings were removed by gentle action with a scalpel and were collected as required. Periodic acid-Schiff/Alcian blue stains were applied to ensure that the scraping process did
not damage the oesophageal tissue. These images can be seen in Figure 3.3 of Chapter 3.

2.3.2 Preparation of oesophageal tissue sections for microscopy

Various microscopic techniques were used within this study. In each case the oesophageal tissue was prepared and sectioned. In order to collect sections as cleanly as possible the slides were initially coated using 3-aminopropyltriethoxysilane (APES). This coating aids retention of tissue sections during the sectioning procedure. The chemicals used in this procedure are listed in Table 2.3. The procedure used is detailed by Maddox & Jenkins (1987) and is outlined below.

Table 2.3. The chemicals used to coat the microscope slides

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decon 90</td>
<td>10 %</td>
<td>Decon</td>
</tr>
<tr>
<td>APES</td>
<td>2 %</td>
<td>Merck</td>
</tr>
<tr>
<td>Industrial methylated spirits</td>
<td>99 %</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

2.3.2.1 APES coating procedure

Brand new microscope slides were soaked in 10 % Decon 90 solution overnight in order to remove any dirt or grease on the slides. The slides were then washed in running tap water via a shallow dish at 60 °C for at least 60 minutes prior to at least two rinses in deionised water. The slides were then dried overnight at 60 °C. It was important that at this stage the slides were clean and sparkling. Any stain or foreign material remaining on them would have affected the coating. The slides were then immersed in a solution of freshly prepared 2 % APES solution in industrial methylated spirits. The slides were then washed in industrial methylated spirits and dried overnight at 37 °C. The slides were then stored and used as usual.
2.3.2.2 Fixation procedure

Oesophageal tissue was frozen rapidly using liquid nitrogen or a freezing fixation spraying agent. Once the tissue was frozen it was cut to an appropriate size and orientated onto section holders for positioning within the cryostat. Cryomatrix embedding medium (supplied by Shandon, USA) was used to fix the sample. The embedded sections were frozen again to aid the sectioning process; liquid nitrogen or spray freezing agents were used for this procedure. The tissue was sectioned using a Shandon E Cryotome cryostat sectioner. This instrument allows frozen tissue to be maintained at low temperatures whilst thin sections are sliced and collected onto APES coated slides. The frozen tissue was stiff and thus retained its shape during the sectioning process. The sections taken were approximately 18 µm thick.

2.3.3 Image analysis used for microscopy

Image analysis software was used in the quantification of the alginate adhesion on oesophageal tissue. The software package used was Image Tool 2.00 and was provided by the University of Texas Health Science Centre in San Antonio (UTHSCSA). The image analysis package was calibrated according to a graticule image. A graticule was imaged under the same conditions as the samples that required measurement. A spatial calibration was collected using the graticule to draw a line of known length. This figure was then set as a standard and the lines drawn were measured in reference to this value. Each image was then captured and lines drawn along the length that required measurement. These lines were drawn in reference to a grid that was placed over the sample image within a second software package, Adobe Photoshop (version 4.0). In each case approximately 20 measurements were recorded and an average value was calculated.
2.4 FORMULATION OF SALIVA USED WITHIN THE EXPERIMENTAL PROCEDURES

As the oesophagus is continually bathed in saliva this fluid may have an important role in the adhesion of a substance onto the oesophagus. This indicates that saliva is an essential material within the investigations performed. However, saliva is a physiological fluid that has a composition that varies in response to many external factors. As this fluid is an important component in this piece of research the variability and steps taken to minimise this diversity are described within this chapter. Mucin is thought to be an important component of saliva when investigating adhesion at interfaces, as rheological synergy between mucin and polymers has been linked to mucoadhesive strength (Mortazavi et al, 1992). Natural saliva, artificial saliva and mucin solutions are discussed below.

2.4.1 Natural saliva

Using natural human saliva within scientific experiments raises several issues associated with the natural variations of the composition of this physiological fluid. The problems associated with the use of saliva are fully discussed in papers by Dawes (1974) and Rudney (1995). Differences associated with age, gender, time of day and even seasonal diversity can affect the composition of saliva and these differences may lead to results that are hard to analyse.

The natural saliva used throughout the study was collected from healthy, non-smoking volunteers at least one hour after eating. All saliva samples were collected as required and used within one hour of collection. Unstimulated saliva was collected from subjects. Saliva was retained within the oral cavity for approximately 60 seconds then ejected into clean vials for immediate use. In ejecting saliva, minimal force was used to prevent collection of excessive oral debris.
2.4.2 Artificial saliva

Artificial saliva provides a physiological medium devoid of the variability of saliva from its natural source. Artificial saliva was used in the retention model and in rheological studies to gain an insight into the interaction between salivary components and alginate solutions. Many different artificial salivas are available commercially and also there are many different formulations quoted in the literature. Comparative studies and evaluations of such formulations have been performed (e.g. Vissink et al, 1983). Table 2.4 compares typical formulations of artificial salivas; a commercially available saliva used for the treatment of dry mouth (British National Formulary, 2000), an ionically formulated artificial saliva based on the values found in the Geigy tables of scientific data (Lentner, 1981) and the artificial saliva formulation listed in the Dental Practitioners’ Formulary (1998).

Table 2.4. Comparison of three different formulations for artificial saliva

<table>
<thead>
<tr>
<th>Commercial saliva (Saliva Orthana®) (per 100ml)</th>
<th>Ionic artificial saliva (per 100ml)</th>
<th>Artificial saliva DPF (per 100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35 g gastric mucin 0.2 g xylitol 0.42 mg sodium fluoride</td>
<td>0.27 g gastric mucin 42 mg sodium carbonate 43 mg sodium chloride</td>
<td>3 g sorbitol 0.65 g carmellose sodium 80.38 mg dibasic potassium phosphate</td>
</tr>
<tr>
<td>Plus preservatives and flavouring agents 0.149 g potassium chloride</td>
<td></td>
<td>62.5 mg potassium chloride</td>
</tr>
<tr>
<td>22 mg calcium chloride 91 mg di-sodium hydrogen orthophosphate</td>
<td></td>
<td>36.62 mg monobasic potassium phosphate 16.62 mg calcium chloride</td>
</tr>
<tr>
<td></td>
<td>5.88 mg magnesium chloride</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.43 mg sodium fluoride</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ preservatives and colours</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4 illustrates the many differences found between formulations of artificial saliva. Commercially available artificial salivas are usually employed to retain moisture within the oral cavity and their ionic formulation has little bearing on natural saliva. Studies that investigate the interactions of dental materials with saliva often use an artificial medium that is ionically similar to natural saliva yet which may have no rheological similarity to the latter (Leung & Darvell, 1997). Levine et al (1987) reviewed many different artificial saliva formulations found in the literature. As yet there is no substitute that mimics both the ionic and rheological properties of saliva; this project would require an artificial saliva that acts in both of these ways.

Two different artificial saliva formulations were chosen from the array available in the literature for this study. The first artificial saliva chosen was based on a formulation used widely within the dental world. Table 2.5 shows the formulation of this artificial saliva (Embleton et al, 1998).

Table 2.5. Formulation of artificial saliva I

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount (g/L)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Lab-Lemco” Powder</td>
<td>1.0</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>5.0</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Hog gastric mucin</td>
<td>2.5</td>
<td>Sigma</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.35</td>
<td>Sigma</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.2</td>
<td>Sigma</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

This solution was heated to 80 °C for 30 minutes prior to addition of 12.5 ml of filter sterilised 40% urea (Sigma).

The second artificial saliva used in the study was formulated based on the ionic environment of natural saliva. This formulation was prepared as per Documenta Geigy handbook (Lentner, 1981). Table 2.6 shows the formulation of this saliva.
Table 2.6. Formulation of artificial saliva II

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount (g/L)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>0.42</td>
<td>BDH</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.43</td>
<td>BDH</td>
</tr>
<tr>
<td>KCl</td>
<td>1.49</td>
<td>BDH</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.22</td>
<td>BDH</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>0.91</td>
<td>BDH</td>
</tr>
<tr>
<td>Porcine gastric mucin</td>
<td>2.70</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(type II)</td>
</tr>
<tr>
<td>Deionised water</td>
<td></td>
<td>to 1000ml</td>
</tr>
</tbody>
</table>

From here onwards the artificial salivas will be referred to as artificial saliva I or II. Artificial saliva II compares favourably to the ionic content of natural saliva although artificial saliva I shows more similar rheological properties. The rheological properties of both artificial salivas and natural saliva are compared in Appendix I.

2.4.2.1 Preparation of artificial saliva

The artificial salivas used were prepared in one-litre batches according to the formulations listed in Tables 2.5 and 2.6. The chemicals were added to deionised water and stirred at a vigorous speed using a magnetic stirrer. The artificial salivas were prepared and used within an eight hour period. When not in use the solutions were stored in a refrigerator.

2.4.3 Preparation of mucin solutions

Mucins or mucoproteins are responsible for the lubricating properties of saliva. They provide saliva with the rheological properties necessary to coat and retain moisture on mucosal surfaces (Tabak et al, 1982). They are usually defined as glycoproteins containing more than 40 % carbohydrate with a protein core and oligosaccharide side chains attached by O-glycosidic linkages. The length of the carbohydrate chain
varies according to the origin of the mucin. Salivary mucins have carbohydrate chain lengths of 6-8 sugars compared to gastric mucins that have 19 sugars per chain (Allen & Pearson, 1993). Mucins are negatively charged due to the ester sulfate and sialic acid residues of the carbohydrate side chains although the protein core also has substantial amounts of acidic amino acids. The shape of mucin molecules has been described as being “bottle-brush”. This is shown schematically in Figure 2.4.

![Figure 2.4](image.png)

*Figure 2.4.* Schematic diagram representing the bottle-brush shape of mucin molecules

The negative charge on the mucin molecules acts to increase the stiffness of this "bottle-brush" structure and leads to a large hydration sphere of the mucin in solution. Cloning of mucin genes has provided much information on the nature of the different structural properties of mucin molecules (Strous et al, 1992).

This study investigates the presence of mucin found within the unstirred water layer resident on œsophageal tissue and also examines the effect that this mucin may have on the interaction between the tissue and the alginate. This area is discussed more fully in Chapter 3, which probes the œsophageal tissue surface.

Within this study, two commercially available mucins (Sigma, UK) were used, porcine gastric mucin (PGM) and bovine submaxillary mucin (BSM). When investigating the interaction between alginate and a mucin solution it is important to consider the chemical properties of the mucin involved. Table 2.7 compares the properties of PGM and BSM.
Table 2.7. Comparison of the composition of porcine gastric mucin and bovine submaxillary mucin

<table>
<thead>
<tr>
<th>Component</th>
<th>PGM %</th>
<th>BSM %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>43.9</td>
<td>57.6</td>
</tr>
<tr>
<td>Neuraminic acid</td>
<td>1.2</td>
<td>6.4</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>16.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Hexose</td>
<td>19.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Mucin chemistry is discussed in further detail in Chapters 3 and 6. Previous studies have shown a difference in the interaction between polymers and mucin according to the type of mucin used (Hagerstrom et al, 2000). This phenomenon will be discussed in greater detail in the relevant experimental chapters.

2.4.3.1 Procedure for the preparation of mucin solutions

Studies were performed investigating the interactions between alginate and solutions of mucin. Crude porcine gastric mucin (type II) supplied from Sigma was used in the studies investigating the interaction between mucin and alginate. This mucin was hydrated in deionised water over a suitable period of time using a magnetic stirrer to aid dispersion. Bovine submaxillary mucin was also investigated in combination with alginate. Solutions of bovine submaxillary mucin were prepared in the same manner as the solutions of porcine gastric mucin.

2.5 STATISTICAL ANALYSES

Statistical analysis was performed on the data to test whether there was a difference in the results noted for one observation as compared to another. For example the retention of one alginate compared to another from the range investigated. In this situation the hypothesis set was that there was a difference in the observation
depending on the alginate investigated. The null hypothesis states that there is no difference in the observation as related to the alginate used. Analysis of variance (ANOVA) tests were performed on the data collected to test the above hypothesis. A computer software package, Statview version 5.0, was used. The significance level was set at 5 % and significant differences were observed when $p < 0.05$.

Analysis of variance, or ANOVA, tests are very similar to the two-sample t-test. ANOVA comparisons are based on the variability of the sample group means about the overall mean relative to the variability of the separate sample observations about their group mean. Thus a mean is calculated for all observations and then also for each group. In short, the greater the differences between categories, relative to the differences within categories, the more inconsistent the data is with the null hypothesis. The ratio of the difference between the sample means compared to the variation within the sample is calculated and the result found termed the F value. This figure relates to the significance of the difference between the two samples according to an F-table. Thus the F value and the degrees of freedom associated with the calculation are taken into account when demonstrating the significance of the relationship. Further details on these techniques can be found in Swinscow (1976).

Linear regression tests were also performed as part of the statistical analyses performed. Linear regression analyses the relationship between two variables, X and Y. A line of best fit is drawn through a set of data points, the equation of this line relates to the linear trend within the sample data. The goal of linear regression is to adjust the values of slope and intercept to find the line that best predicts Y from X. The regression of this data relates to the relationship between the linear equation and the real data observed. $R^2$ is the symbol used to represent the goodness of fit of linear regression. The value $R^2$ is a fraction between 0.0 and 1.0, and has no units. An $R^2$ value of 0.0 means that knowing X does not help to predict Y. There is no linear relationship between X and Y, and the best-fit line is a horizontal line going through the mean of all Y values. When $R^2$ equals 1.0, all points lie exactly on a straight line with no scatter. Knowing X lets you predict Y.
Chapter Three: Investigation into the oesophageal surface layer

CHAPTER 3: INVESTIGATION INTO THE OESOPHAGEAL SURFACE LAYER

3.1 INTRODUCTION

When investigating the adhesion of alginate solutions to oesophageal tissue it is important to examine the tissue surface properties. Chapter 1 described the presence of an unstirred water layer that resides on the surface of oesophageal tissue. This chapter describes biochemical and histological techniques used to probe the composition of this layer. Histological staining was applied to the tissue surface to detect mucin within the unstirred water layer. Biochemical analyses were undertaken to evaluate the concentration of mucin within this same, important layer. Identical analytical techniques were also applied to human saliva in order to determine the mucin concentration. The rationale behind the investigation into human saliva lies in the theory that the unstirred layer may be derived from swallowed saliva.

3.2 COMPOSITION OF THE UNSTIRRED WATER LAYER

Very little work has been performed on the characterisation of the unstirred water layer as noted in Chapter 1. The depth of the unstirred water layer has been previously suggested to be 30 μm (Attwood, 1994), although (Sarosiek et al, 1993) suggested a thicker layer at 95 μm. A pH gradient of 1-2 units has also been measured across the unstirred water layer (Edwards, 1984). Within this chapter oesophageal tissue was scraped in order to further analyse the unstirred water layer. The term oesophageal scrapings refers to the unstirred water layer material removed from the tissue surface. This removal procedure was described in Chapter 2.

3.2.1 Mucin within the unstirred water layer

Controversy surrounds the presence of a mucus layer resident on oesophageal tissue. Nunn et al (1990) demonstrated the presence of mucus on the surface of the rat stomach and intestine, yet only bacteria were shown to be present on the oesophageal surface.
Namiot et al (1994) have demonstrated a putative mucus layer on human oesophagus and Tanaka et al (1996) have shown mucus to be present on rat oesophagus. Dixon (1997) has proved the existence of and analysed the mucin type material present on porcine oesophageal tissue.

A brief overview of the mucins used within this study was supplied in Chapter 2, however, more detail about mucin is provided within this introduction. Mucus is the major organic secretion of the gastro-intestinal tract from the stomach to the colon. It is secreted as a gel that adheres to the epithelial surfaces and acts to protect the underlying cells from damage caused by aggressive chemicals or the passage of food (Allen, 1989). The mucus gel is comprised of 90-95% water, by weight. Other components present within the mucus gel include salts, proteins, nucleic acids, carbohydrates and lipids. The mucus layer has been quantified within the human and rat stomach and is said to be 180 and 73 μm thick respectively (Allen et al, 1990; Kerss et al, 1982). However, no mucus layer has been observed within the oesophagus. Although a continuous mucus layer is lacking in the oesophagus, mucin has been detected within this organ in previous studies (Namiot et al 1994b; Dixon 1997). However, the source of this mucin has not been determined. Oesophageal mucin may be derived from oesophageal secretions.

Carbohydrate contributes between 50 – 90% to the weight of the mucin glycoprotein in the form of oligosaccharide side chains. These side chains can be branched or linear and the number of sugars in a particular chain can vary from a simple two through to nineteen (Allen, 1981). Mucins are negatively charged due to ester sulphates and sialic acid residues. The negative charge increases the stiffness of the "bottle-brush" regions and is the basis of different histological staining for acidic and neutral mucins (Allen & Pearson, 1993). Neutral mucins are readily detected by periodic acid-Schiff staining whereas acidic residues are determined by Alcian blue staining (Filipe 1979).

The mucus layer has been quantified within the human and rat stomach and is said to be 180 and 73 μm thick respectively (Allen et al, 1990; Kerss et al, 1982). However, no mucus layer has been observed within the oesophagus. Although a continuous mucus layer is lacking in the oesophagus, mucin has been detected within this organ in previous studies (Namiot et al 1994b; Dixon 1997). However, the source of this mucin has not been determined. Oesophageal mucin may be derived from oesophageal secretions.
and/or swallowed saliva. The composition of this mucin is noted in Table 3.1. The table compares the amino acid content of porcine oesophageal mucin (POM) to the two mucins present in saliva, (MG1 and MG2) as well as commercially available bovine submaxillary mucin (BSM) and porcine gastric mucin (PGM).

**Table 3.1.** A comparison of the composition of MG1, MG2 (Loomis et al, 1987), BSM (Wu et al, 1994), POM and PGM (Dixon, 1997)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>MG1</th>
<th>MG2</th>
<th>BSM</th>
<th>POM</th>
<th>PGM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>0.66</td>
<td>0.30</td>
<td>0.10</td>
<td>0.54</td>
<td>0.36</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.63</td>
<td>1.29</td>
<td>0.76</td>
<td>0.53</td>
<td>0.95</td>
</tr>
<tr>
<td>Serine</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.86</td>
<td>0.43</td>
<td>0.37</td>
<td>0.72</td>
<td>0.51</td>
</tr>
<tr>
<td>Proline</td>
<td>0.92</td>
<td>1.45</td>
<td>0.55</td>
<td>0.41</td>
<td>0.87</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.83</td>
<td>0.09</td>
<td>0.88</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.78</td>
<td>3.14</td>
<td>0.61</td>
<td>0.30</td>
<td>0.37</td>
</tr>
<tr>
<td>Half-cysteine</td>
<td>0.23</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Valine</td>
<td>0.61</td>
<td>0.19</td>
<td>0.33</td>
<td>0.30</td>
<td>0.47</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.10</td>
<td>0.01</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.26</td>
<td>0.09</td>
<td>0.08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.71</td>
<td>0.18</td>
<td>0.18</td>
<td>0.21</td>
<td>0.57</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.23</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.28</td>
<td>0.05</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.28</td>
<td>0.09</td>
<td>0.03</td>
<td>0.27</td>
<td>0.17</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.21</td>
<td>0.05</td>
<td>0.01</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.34</td>
<td>0.04</td>
<td>0.21</td>
<td>0.34</td>
<td>0.24</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.70</td>
<td>0.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Porcine oesophageal mucin is compared to salivary and gastric mucins as a means of determining the source of this mucin present within the oesophagus. This table
demonstrates the fact that the porcine oesophageal mucin as analysed was not noticeably similar to any of the other mucins characterised. This result suggests that oesophageal mucin is derived from sources not covered by the table or that it is a unique material specific to the oesophagus. The importance of the two salivary mucins is explained later in this chapter.

There is no commercially available product that can be used to mimic the properties of the substances found naturally on oesophageal tissue. This study used commercially available porcine gastric and bovine submaxillary mucin as an alternative source. The mucin content within the unstirred water layer was measured using a colorimetric assay based on the periodic acid–Schiff (PAS) reaction.

The PAS stain is used histologically as a specific stain for carbohydrates. This chemical interaction was developed as an assay technique to assess the glycoprotein content of a given sample. The technique was described by Mantle (1979) although an outline is provided below. The technique has two parts; initially the glycol groups on the glycoprotein are converted to reactive aldehydes by the action of periodic acid; and then Schiff's reagent colours them bright magenta. The experiment is performed as a two-step process as Schiff's reagent must be stored separately to the acid component. If Schiff's reagent were added directly to the acid a red colour would develop due to the non-specific oxidation of the unbound fuchsin. Schiff's reagent is prepared by the addition of hydrochloric acid and sodium metabisulfite to basic fuchsin. The sodium metabisulfite acts to prevent oxidation of the fuchsin component on storage yet must react with the excess periodic acid on the addition of the two materials. The basic chemistry is shown in Figure 3.1.

Hydrochloric acid and sodium metabisulfite are added to discolour the basic fuchsin from red to colourless. Once the oxidised glycoprotein (mucin) was added, the solution became red again indicative of the fuchsin-glycoprotein complex. Only one sulfonic acid group needs to complex with the oxidised glycoprotein to produce the conjugated chromophore. The absorbance for any given glycoprotein will depend directly on the number of oxidisable hydroxyl groups present on the molecule. The absorbance will also depend upon the purity of the sample in question. In a molecule of glycogen every sugar residue is oxidisable. However, other glycoproteins may exhibit less colouration
due to glycosidic linkages that may prevent oxidation of certain sugar residues. Certain residues including sialic acid lead to higher colouration values. This needs to be considered when performing a colorimetric assay. Table 3.2 compares the sialic acid content of pig oesophageal mucin, pig submaxillary mucin and pig gastric mucin (Dixon, 1997).

Figure 3.1. The basic chemistry involved in the PAS assay
Table 3.2. Comparison of the sialic acid content in pig œsophageal, submaxillary and gastric mucin

<table>
<thead>
<tr>
<th></th>
<th>Pig œsophageal mucin</th>
<th>Pig submaxillary mucin</th>
<th>Pig gastric mucin</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Sialic acid by weight</td>
<td>8.25</td>
<td>15.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

This table shows the variability noted between mucin located in different areas. As stated the absorbance noted from the PAS assay is dependant upon the oxidisable hydroxyl groups plus the number of sialic acid groups present. Thus an assay based on the mucins above would give different colouration levels at similar concentrations due to the different chemical composition of the materials. It is important in quantifying the mucin content of an unknown sample to use a control. The data above highlights the necessity for a control that exhibits the same profile as the unknown mucin in question. However, porcine œsophageal mucin is not available commercially, thus this study used isolated human salivary mucin, bovine submaxillary mucin and porcine gastric mucin as comparative controls in determining the mucin content of the unstirred water layer.

3.3 TECHNIQUES USED THE ANALYSIS OF THE UNSTIRRED WATER LAYER

The techniques used revolved around the determination and characterisation of mucin within the unstirred water layer. These techniques included histological staining, isolation and characterisation of salivary mucin and a periodic acid-Schiff assay for the determination of glycoprotein content. These techniques enabled the mucin present within the unstirred water layer to be visualised and quantified. The depth of the unstirred water layer was also assessed using two different techniques.

3.3.1 Measurement of the depth of the unstirred water layer

Porcine œsophageal tissue was taken and scraped over an area of 30 by 30 mm. The mass of scrapings collected was determined using an Oertling NA264 balance. The
density of the scrapings was assumed to be equal to 1 $g/mm^3$ and thus the depth of the unstirred water layer could be calculated.

3.3.2 PAS/Alcian blue staining procedure

The PAS/Alcian blue staining technique highlights the glycoprotein content in tissue sections, particularly mucous components. The procedure used is taken from Jordan et al (1998). An outline of this procedure is detailed below. This staining procedure was applied to porcine gastric and porcine oesophageal tissue sections. Table 3.3 provides a full inventory of the chemicals used in the staining technique.

Table 3.3. Chemicals used in the PAS/Alcian blue staining procedure

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>100 %</td>
<td>Sigma</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>3 %</td>
<td>Sigma</td>
</tr>
<tr>
<td>Alcian blue</td>
<td>1 % in 3 % acetic acid</td>
<td>Searle diagnostic</td>
</tr>
<tr>
<td>Periodic acid</td>
<td>1 %</td>
<td>Sigma</td>
</tr>
<tr>
<td>Schiff's reagent</td>
<td>As supplied</td>
<td>Sigma</td>
</tr>
<tr>
<td>Sodium metabisulfite</td>
<td>0.5 % in deionised water</td>
<td>Sigma</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Gelatine</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>• gelatine</td>
<td>10 g in 60 ml deionised water</td>
<td></td>
</tr>
<tr>
<td>• glycerol</td>
<td>70 ml</td>
<td>Sigma</td>
</tr>
<tr>
<td>• phenol crystals</td>
<td>250 mg</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Tissue was prepared and sectioned as described in Chapter 2. These prepared slides were placed in a domestic freezer at -18 °C overnight to fix the sample to the slide. The slides were thawed for 20 minutes prior to beginning the staining process. They were then submerged in 100 % ethanol for 10 minutes to pre-fix the sections. The slides were then washed gently in running tap water for 10 minutes before equilibrating in 3 % acetic acid for 2 minutes. This was followed by a staining stage in which the slides
were immersed in Alcian blue dissolved in 3 % acetic acid for 2.5 hours. In order to clean the slides they were placed in 3 % acetic acid for 2 minutes then rinsed in running tap water for a further 2 minutes. The next stage was to place the slides in distilled water for 2 minutes. This was followed by immersion in 1 % periodic acid for 10 minutes and then a 5 minute rinsing process in running tap water. The next staining agent was Schiff’s reagent. The slides were submerged for 15 minutes in Schiff’s reagent and then washed for 2 minutes in running tap water. The final stages rinsed and set the stained sections on the slides; three rinses of 1 minute each were performed in 0.5 % sodium metabisulphite, followed by rinsing for 5 minutes in running tap water. The final stage, which dried the slides was fixation using paraformaldehyde vapour for 45 minutes at 37 °C. Paraformaldehyde is a fixing agent that cross-links the carbohydrate moieties of the specimen and permanently attaches them to the slide (Bancroft & Cook, 1994). The slides were removed from the paraformaldehyde vapour and then preserved by mounting in warm gelatine and covering with a cover slip. The slides were then kept at room temperature. Photographs of the slides were taken using a Nikon HFX-II light microscope and ISO 200 speed Kodak colour film.

3.3.3 Isolation of human salivary mucin

The mucin concentration within human saliva has previously been determined by Navazesh et al (1992). However, the results were quoted as mucin dye-binding units per millilitre rather than concentration values. Similar reports were made by Denny et al (1991). Sarosiek et al (1994) quoted a basal rate of mucin output as 0.24 mg/minute however, the total saliva flow rate was not given. Previous work performed within the aforementioned group (Namiot et al, 1994a) showed the basal saliva flow rate to be 0.36 mg/minute. Calculations show that using these values the basal mucin output is 0.66 mg/ml. This value can be expressed as a percentage value weight by volume of 0.066 % w/v. Geigy tables do not express mucin as a concentration within whole saliva. However, the dry mass of the saliva is quoted as only 0.6 % w/v (Lentner, 1981), thus the mucin concentration must be lower than 0.6 %. Thus the literature values are very diverse and the values quoted for the mucin concentration in saliva vary by a factor of ten.
In this study human salivary mucin was isolated and characterised. This material was then used as a standard in the preparation of a calibration curve for use in the determination of mucin concentration within an unknown sample of both human saliva and esophageal scrapings. Two distinct mucins are present in saliva and they are termed MG1 and MG2. These mucins have previously been isolated and characterised (Nielson et al, 1997). MG1 is a high molecular weight mucin with the oligosaccharide chains distributed unevenly along the peptide chain. MG2 is of a lower molecular weight with the side chains distributed evenly along the length of the peptide chain (Quissell & Tabak, 1989). MG1 is characterised by a covalent stabilisation of its suprastructure via disulfide bonds. In contrast, MG2 forms a suprastructure via non-covalent interactions. MG2 has been assigned as the product of the MUC7 gene and MG1 the MUC5B gene (Mehrotra et al, 1998). Table 3.1 compared the chemical composition of the two salivary mucin moieties.

### 3.3.3.1 Procedure for the isolation of human salivary mucin

Human saliva was collected from healthy volunteers over a three hour time period and pooled into a protease inhibitor buffer solution kept on ice. This saliva/buffer solution was stirred gently using a magnetic stirrer. The unstimulated saliva was stored within the oral cavity for up to 60 seconds then ejected into a collection vial. The forces used in the ejection process were small in order to minimise the presence extraneous of unwanted oral debris. Table 3.4 shows the formulation of the protease inhibitor buffer used (Pearson & Mason, 1977).
Table 3.4. Protease inhibitor formulation used in the collection of human saliva

<table>
<thead>
<tr>
<th>Amount (g)</th>
<th>Chemical</th>
<th>Rationale for inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0185</td>
<td>Iodoacetamide</td>
<td>Inhibits thiol dependant proteinases</td>
</tr>
<tr>
<td>1.312</td>
<td>$\alpha$-(6)-aminocaproic acid</td>
<td>Inhibits the activation of plasminogen to plasmin</td>
</tr>
<tr>
<td>0.372</td>
<td>EDTA</td>
<td>Inhibits metalloependant proteinases</td>
</tr>
<tr>
<td>0.125</td>
<td>N-ethyl maleiamide</td>
<td>Inhibits thiol dependant proteinases</td>
</tr>
<tr>
<td>0.235</td>
<td>Benzamidine hydrochloride</td>
<td>Specific inhibitor for trypsin and trypsin-like enzymes</td>
</tr>
<tr>
<td>0.052</td>
<td>Phenylmethylsulfonylfluoride</td>
<td>Inhibits serine proteases</td>
</tr>
<tr>
<td></td>
<td>dissolved in propanol</td>
<td></td>
</tr>
</tbody>
</table>

This should be made up to 100ml using 1:10 salt azide (supplied by BDH) stock solution. The final two ingredients should be added immediately prior to use.

Approximately 20 ml of the buffer solution was placed on ice. This volume was added throughout the collection period to prevent degradation of the collected saliva. Once a volume greater than 50 ml of saliva had been collected, the saliva/inhibitor solution was centrifuged at 9000 rpm for 60 minutes on an Eppendorf 5810-R centrifuge. This separated the soluble salivary components including mucin from the insoluble solid food matter and cell debris. The supernatant was retained and the mucin component was purified by density gradient ultracentrifugation in caesium chloride as reported by Sarosiek et al (1984) and described briefly below. The density of the supernatant was adjusted to a density value between 1.415 and 1.425 g/ml by the addition of solid caesium chloride. This is the density range of mucin and provides a means of separating the mucin from other soluble saliva components such as lipids and fatty acids (Starkey et al, 1974). During the ultracentrifugation process the soluble matter was separated according to density within the centrifugation vessel. The centrifugation took place using a Centrikon T-2060 ultracentrifuge at 40000 rpm ($1.5 \times 10^5$ g) at 4 °C. The resulting material was separated, by density, into nine equal fractions, each of which was exhaustively dialysed against deionised water to remove the high concentrations of caesium chloride. At least nine water changes were made over a 12-hour period. A
periodic acid–Schiff (PAS) assay, described in detail later in this chapter was performed on each of the fractions using papain digested porcine gastric mucin as a standard. Only those fractions that showed a mucin content of greater than 25 μg in the total 200 μl sample were retained for purification. The mucin was purified from the relevant fractions by freeze-drying the selected solutions.

The collected, freeze-dried mucin was analysed using gel filtration chromatography. This technique was described in Chapter 2 in reference to the preparation of the covalently labelled alginate solution. 2 mg of the freeze-dried sample of isolated mucin was dispersed in 1ml of a 1:10 salt azide solution. This solution was run down a pre-calibrated Sepharose CL-2B column and column fractions of 1ml were collected between the V₁ and V₀ of the column. The column dimensions were 30 cm length and 20 mm diameter. 500 μl aliquots of every other column fraction were diluted one in two in water and tested for glycoprotein activity using the PAS assay described later in this chapter. The results are shown in the section 3.4.3 of this chapter.

3.3.4 Periodic acid - Schiff assay for the analysis of glycoprotein content.

A periodic acid-Schiff assay (PAS) was used to determine the mucin content within both natural saliva and the unstirred water layer resident on oesophageal tissue. This technique involves a colourimetric assay to determine the glycoprotein content of a given sample. Mucin is the predominant glycoprotein present in saliva, thus this assay acts to provide an accurate assessment of the mucin content present in saliva and oesophageal scrapings. The method was described by Mantle (1978). The materials used are listed in Table 3.5.

Table 3.5. Chemicals used in the PAS assay.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium metabisulfite</td>
<td>0.1 g in 6 ml Schiff’s reagent</td>
<td>Sigma</td>
</tr>
<tr>
<td>Schiff’s reagent</td>
<td>6 ml</td>
<td>Sigma</td>
</tr>
<tr>
<td>Periodic acid</td>
<td>20 μl in 10 ml 7 % acetic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Acetic acid (granular)</td>
<td>0.7 g in 10 ml deionised water</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
3.3.4.1 PAS assay procedure

Aqueous samples of mucin containing material were obtained. A 200 µl aliquot of each aqueous sample was taken and made up to 2 ml with deionised water for analysis. 200 µl of freshly made periodic acid solution was added to each sample and the samples were mixed then incubated at 37 °C for 60 minutes. The sodium metabisulfite in Schiff's reagent was also incubated at 37 °C for 60 minutes. 200 µl of this solution was added to the incubated sample tubes. These tubes were then mixed prior to further incubation at room temperature for 30 minutes. This second incubation period allowed the reaction to occur and the colour to develop. Each sample was measured at an absorbance of 555 nm against a blank. The glycoprotein content in these samples was calculated against a standard curve. A new standard curve was produced for each experiment, as the absorbance measured was dependant on both the time of equilibration and also the reagents used. Consequently, fresh reagents were made up for each experiment.

3.3.4.2 Preparation of a standard curve

In order to produce a standard curve, either papain digested porcine gastric mucin, bovine submaxillary mucin or purified human salivary mucin were used. A 1 mg/ml aqueous solution of the mucin was prepared. Standards containing 0, 25, 50, 75, 100 and 150 µl of this solution were prepared and each one made up to 2 ml using deionised water. The standards were taken through the PAS procedure as described above. The absorbance values obtained for the standard samples were plotted against mucin content and a line of best fit was drawn through these points. The equation of the line gives a formula that was used to determine the mucin content of the unknown samples under measurement. Figure 3.2 shows an example of a standard curve obtained for papain digested porcine gastric mucin.
Figure 3.2. A standard curve produced using porcine gastric mucin

The equation of the line of best fit as shown on the graph can be used to calculate the mucin concentration of unknown samples. A standard curve comparing PGM, BSM and isolated human salivary mucin was prepared. The regression value as shown on the graph was greater than 0.9 indicating a very strong trend linking mucin concentration to the absorption of the sample. A standard curve producing a regression value less than 0.9 was rejected and the experiment repeated.

The mucin content of saliva collected from 4 volunteers was measured using isolated human salivary mucin as a standard. This experiment investigated the inter-individual mucin content within unstimulated saliva samples. The mucin content of one volunteer's saliva was measured on two separate occasions in order to note the variation between collected samples. 2 ml of saliva was collected from each individual and split into two 1.5 ml Eppendorf tubes and centrifuged for 60 minutes at 2000 rpm using a Sigma 3K10 centrifuge. The resulting supernatent was kept for analysis and the insoluble debris collected as a pellet was discarded. 200 µl of each sample was analysed according to the PAS assay.

The mucin concentration within the unstirred layer was also investigated. Oesophageal scrapings were removed from oesophageal tissue as described in Chapter 2. 0.677g of scrapings were collected and this material was dispersed into 2 ml deionised water, thus the concentration of scrapings was 0.3385 g/ml. The suspension was then separated into two 1.5 ml Eppendorf tubes and centrifuged using the same method and
instrumentation as for the collected saliva samples described above. This centrifugation separated the soluble matter from the insoluble cellular debris. A 200 µl aliquot from each of the resulting solutions was taken and a PAS assay performed.

3.4 RESULTS AND DISCUSSION

A histological staining technique using Alcian blue and Schiff’s reagent was applied to porcine oesophageal tissue sections. These techniques stain glycoprotein moieties present within the tissue section. The mucin content of both natural saliva and scrapings taken from porcine oesophageal tissue were measured using a colourimetric assay techniques based on the periodic acid- Schiff reaction (PAS).

3.4.1 Calculation of the depth of the unstirred water layer

Scrapings were taken from a 900 mm² area of porcine oesophageal tissue and the mass of the scrapings determined. This mass was correlated to a depth measurement for the unstirred water layer, assuming the density to equal 1 g/ml. An example calculation is shown below.

**Step One**

The mass collected was measured to be X g

**Step Two**

This mass was converted into a volume assuming the density to equal 1g/ml.

\[ X \text{ g} = X \text{ ml} \]

Thus \( X \text{ g} = 1000.X \text{ mm}^3 \)

**Step Three**

The depth of the layer was calculated by dividing the volume collected over the area of the tissue scraped

\[ \frac{1000.X \text{ mm}^3}{900 \text{ mm}^2} = \text{ the depth of the layer (mm)} \]

This depth value was converted to µm due to the magnitude of the value.
Eight pieces of oesophageal tissue were used within the study and the average depth of the unstirred water layer was calculated to be 107.3 (± 30) µm. This value correlates with a previously quoted value of 95 µm for the depth of the unstirred water layer (Sarosiek et al, 1993).

3.4.2 PAS/Alcian blue staining procedure

Natural and scraped porcine oesophageal tissue as well as porcine gastric tissue sections were taken and stained using periodic acid-Schiff (PAS) and Alcian blue staining techniques. This study was performed in order to characterise the unstirred water layer and to visually detect mucin components. Gastric tissue was also investigated to observe the difference in the protective layers present on these two tissue types. Figure 3.3 compares the images of natural (unscraped) and scraped porcine oesophageal tissue.

(a) Unscraped tissue

(b) Scraped tissue

Figure 3.3. PAS/Alcian blue staining applied to both natural and scraped tissue. The scale bar represents 1000 µm.
This image shows the mucous components of the oesophageal tissue. As well as staining mucin components a blue colour; basal lamina, nuclei and other cellular material was also coloured. Figure 3.3 (a) above showed a clear stained edge to the tissue on the luminal side. This colour was the result of mucin components present on the surface of the tissue section. This distinct layer was not present in the scraped tissue suggesting that this was removed during the scraping process. It was interesting to note that the remainder of the tissue section was unaffected by the scraping process. A certain degree of staining was present on Figure 3.3 (b), thus it can be concluded that many mucin components present in the uppermost layer were removed by scraping the tissue surface. However, there are still some mucin components present on the scraped tissue surface.

Image analysis was performed on the images shown and the depth lines (yellow) were measured and analysed. The analysis performed was described in Chapter 2. Thus the depth of the surface layer on scraped tissue was calculated to be 19.97 (± 7.3) μm (n=19). The natural tissue exhibited a surface depth value of 53.01 (± 33.5) μm (n=18). The depth of the unstirred water layer removed by the scraping process was calculated by a simple subtraction procedure. This removed unstirred layer was calculated to measure 33.04 μm thick. The thickness of the unstirred water layer has previously been suggested as being 30 μm (Attwood, 1994) or 95 μm thick (Sarosiek et al, 1994). The result found using this procedure was in good agreement with Attwood (1994).

The staining procedure was applied to porcine oesophageal tissue and porcine gastric tissue. This study was performed in order to compare the thickness of the surface layer present in the oesophagus with the gastric mucus layer. Figure 3.4 shows the result.
Figure 3.4. Comparison of PAS/Alcian blue staining applied to (a) porcine oesophageal and (b) porcine gastric tissue. Scale bar represents 1000 µm.

The thick mucus layer present on gastric tissue is stained a much richer colour than the mucous components present within the unstirred water layer resident on oesophageal tissue. This is due to the proportionally higher concentration of mucin within the gastric mucus layer as compared to the unstirred water layer resident on oesophageal tissue. The gastric layer is also considerably thicker than the unstirred water layer on oesophageal tissue. Image analysis was again performed on these two tissue sections and the average depth of the mucous layer was calculated. The average depth of the mucous layer on gastric tissue was calculated as being 566.22 (±115.9) µm (n=21). This value is typical of the depth of the mucus layer in the fundic region of the porcine gastric tissue examined (Allen, 2000). The depth of the unstirred water layer resident on oesophageal tissue was calculated via microscopy to be 35.04 µm. This demonstrates the immense depth difference between the gastric mucus layer and the oesophageal unstirred water layer.
3.4.3 Isolation and characterisation of human salivary mucin

Human salivary mucin was collected according to the method described previously in section 3.3.3. Gel filtration chromatography was performed on this collected material as a means of assessing the purity of the mucin. This technique separates material according to molecular size. As discussed previously human saliva comprises of two distinct mucin entities. These mucins have different molecular weights with MG1 being high and MG2 low molecular weight. A 2 mg/ml solution of the collected material was run down a precalibrated gel filtration column. Figure 3.5 shows the data collected.

The results show two distinct peaks, these peaks relate to the two mucin moieties present in human saliva. The peaks observed were distinct and sharp indicating that there was little impurity within the sample. The high molecular weight mucin, MG1, was represented by the initial peak and the low molecular weight mucin, MG2 by the second peak. This result confirms that the material collected was salivary mucin and this test also demonstrated the purity of the collected material.

![Figure 3.5. Separation of the two mucin moieties present in human saliva](image)

3.4.4 Periodic acid-Schiff analysis for the detection of glycoprotein

PGM, BSM and human salivary mucin (HSM) were compared in the preparation of a standard curve. These curves show the absorption of a solution containing a known
amount of mucin. They are used as comparative control data for the calculation of mucin within unknown samples. This comparative curve can be seen in Figure 3.6.

**Figure 3.6.** Comparison of the standard curves for porcine gastric mucin (PGM), purified human salivary mucin (HSM) and bovine submaxillary mucin (BSM)

It can clearly be seen that the absorbance found when using PGM as a standard was far greater than that found when using BSM or HSM. The diversity noted may be due to the inherent variability between the different mucins used. As stated previously, high sialic concentration leads to greater colouration. Table 3.2 showed that the sialic acid concentration in submaxillary mucin is greater than in gastric mucin. This difference may account for the differences in the absorbance values noted. This has implications in the testing of material where the appropriate mucin is not available to perform a standard curve. The standard is used to quantify the mucin present within an unknown sample. According to the reference used, as observed in Figure 3.6, the concentration determined may be greatly affected by the standard used. A suitable standard is therefore necessary in the determination of the mucin content of an unknown sample. In the determination of the mucin content of human saliva an obvious standard was available and isolated human saliva was used. As it is time consuming to prepare isolated human salivary mucin this result shows that commercially available bovine submaxillary mucin may be substituted and the results found would be far more comparable than those attained using porcine gastric mucin as a standard. In the calculation of the mucin content in oesophageal scrapings there is no obvious standard and thus the values found for the three standards measured here were compared.
3.4.4.1 Determination of the mucin concentration within oesophageal scrapings

Histological staining of oesophageal porcine tissue revealed the presence of mucin components on the epithelial surface. A PAS assay was then performed on oesophageal scrapings removed from the tissue in order to quantify the mucin components.

The mucin content of porcine oesophageal scrapings was calculated against the PGM, BSM and HSM standards. The results are shown in Table 3.6.

**Table 3.6.** Concentration of mucin detected in each sample of oesophageal scrapings

<table>
<thead>
<tr>
<th>Standard</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine Gastric Mucin</td>
<td>371.71</td>
<td>434.87</td>
<td>403.29</td>
</tr>
<tr>
<td>Human Salivary Mucin</td>
<td>781.39</td>
<td>914.72</td>
<td>848.06</td>
</tr>
<tr>
<td>Bovine Submaxillary Mucin</td>
<td>903.34</td>
<td>1063.36</td>
<td>983.34</td>
</tr>
</tbody>
</table>

Table 3.6 provides the mucin concentration found in the dispersion of scrapings analysed. This value can be converted to the percentage of mucin present in the scrapings by the procedure outlined below. The porcine gastric mucin average value will be used as an example.

**Step 1**

Convert the mass/ml from µg to g = Multiply by $10^{-6}$

$= 4.03 \times 10^{-4}$

The mass of scraping per ml was calculated to be 0.3385 g/ml

**Step 2**

Set this value to 100 % $= 100/0.3385$

$= 295.42$
Step 3

Multiply the amount of mucin by the value found above to determine the % mucin in the sample

\[ = 4.03 \times 10^{-4} \times 295.42 \]

\[ = 0.119 \% \]

Table 3.7 shows the average values for the three standards of the mucin concentration present in the oesophageal scrapings as a percentage weight by weight (w/w) value.

\[ \text{Table 3.7. } \% \text{ w/w mucin content of oesophageal scrapings} \]

<table>
<thead>
<tr>
<th></th>
<th>Porcine Gastric Mucin</th>
<th>Human Salivary Mucin</th>
<th>Bovine Submaxillary Mucin</th>
</tr>
</thead>
<tbody>
<tr>
<td>% w/w mucin content</td>
<td>0.12</td>
<td>0.25</td>
<td>0.29</td>
</tr>
</tbody>
</table>

This data suggests that the mucin within the unstirred water layer resident on oesophageal tissue was present in very low quantities. The thickness of the unstirred water layer was previously calculated to be 35.04 μm thick. Assuming a continuous mucin presence within the unstirred water layer at 35 μm thick; the continuous layer would measure less that 0.3 % of the unstirred water layer thickness (data from Table 3.7 above). This calculation suggests that the thickness of the mucin layer would be less than 0.1 μm over the oesophageal tissue surface. Dixon (1997) determined that the mean yield of mucin collected from porcine oesophageal tissue was 0.47 μg/cm². This averages a mucus thickness of less than 0.1 μm over the oesophageal tissue. The results found here are in agreement with the results found by Dixon (1997).

3.4.4.2 Determination of the mucin content in human saliva

Analysis of the mucin content in saliva aids this project in many ways. Saliva is a physiologically variable fluid and as such certain analyses aid the understanding and discussion of results found in later stages. Mucin is believed to play an important role in adhesive systems, thus characterisation of the substrates involved in the interaction
assisted in the understanding of the adhesive phenomenon. As noted previously the mucin concentration within human saliva was quoted to be 0.066 % w/v by one group (Sarosiek et al, 1994; Namiot et al 1994a) although the Geigy tables suggest the value may be as high as 0.6 %w/v (Lentner, 1981). Knowledge of the mucin content present in saliva provides a useful back up to other experiments.

Saliva was collected from 4 volunteers and a PAS assay performed to analyse the mucin concentration within each saliva sample. Isolated human salivary mucin was used as a control. Table 3.8 shows these results.

Table 3.8. Comparison of the concentration of mucin in the saliva of four volunteers

<table>
<thead>
<tr>
<th>µg/ml mucin in the sample</th>
<th>% mucin w/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteer 1</td>
<td>485.6 ± 26.4</td>
</tr>
<tr>
<td>Volunteer 2</td>
<td>1888.3 ± 148.6</td>
</tr>
<tr>
<td>Volunteer 3</td>
<td>1014.7 ± 208.3</td>
</tr>
<tr>
<td>Volunteer 4</td>
<td>2389.7 ± 477.8</td>
</tr>
<tr>
<td>Average</td>
<td>1444.6</td>
</tr>
</tbody>
</table>

Table 3.8 shows a great deal of variability between the concentration values of mucin present in the collected saliva. The range noted for mucin concentration in human saliva is outside the values quoted in the literature. However, the values found in the literature varied by a factor of one thousand and as such a "real" estimation of the mucin concentration within saliva was not available. The results shown were inherently variable although this may be explained by the natural variability associated with saliva and also the small population size investigated.

A second study was performed to analyse the mucin content of one volunteer's saliva on two separate occasions. The results found that the samples collected differed sample 1 showed a mucin content of 485.6 µg/ml (± 26.4) or 0.49 % w/v whereas sample 2 showed a mucin concentration of 746.6 µg/ml (± 67.0) or 0.75 % w/v. The time period between the collection of these two samples was 48 hours although each sample was
measured independently. Again this difference in values determined for the mucin concentration in human saliva may be attributed to the natural variability associated with human saliva. It was interesting to note that the variability between one volunteer's saliva on two separate occasions was less than that noted in an interindividual study.

3.5 CONCLUSIONS

The depth of the unstirred layer was calculated using both microscopic and mass analysis techniques. The depth was calculated to be 107.3 μm using mass analysis and 33.04 μm using microscopy. These results were found to be significantly different using analysis of variance (ANOVA) tests at a significance level of 5 %, p < 0.05. However, previous work has suggested that the depth unstirred water layer was 30 μm (Attwood, 1994) and 95 μm (Sarosiek, 1994). The values determined within this study correlate well to those found in previous studies.

PAS/Alcian blue staining techniques exhibited the presence of mucin within the unstirred water layer. This mucin was quantified and found to be present at a concentration of less than 0.3 % w/w. This value correlates well with a previously determined value suggested by Dixon (1997). The mucin content in saliva was also determined at a range from 0.5 – 2.4 % w/v.
CHAPTER 4: RETENTION MODEL

4.1 INTRODUCTION

Adhesion within biological systems has been defined as the ability of a material (synthetic or biological) to adhere to biological tissue for an extended period of time (Duchene et al, 1988). Within drug delivery research the advantages of a bioadhesive system include increasing the residence time of the adhesive formulation, thus increasing drug absorption as well as reducing dosage frequency. The second advantage relies on the fact that bioadhesive formulations make close contact with the underlying mucosal tissue thus the concentration gradient of the drug is steep and clearance of the drug is reduced (Junginger, 1990; Harris & Robinson, 1990). Chapter 3 identified the composition of the biological substrate to which the adhesive formula is targeted. Mucus was shown to be present on the oesophageal tissue surface so the term mucoadhesion rather than bioadhesion is applicable within this situation.

Many techniques and model systems have been employed as a means of assessing the mucoadhesive strength of a variety of polymeric formulations. All such formulations are believed to have potential in the development of a mucoadhesive drug delivery system. Many tests focus on the measurement of the interaction strength between a bioadhesive formulation and a biological substrate. There are two thorough reviews that detail the nature of such tests (Helliwell, 1993; Jimenez-Castellanos et al, 1993).

This study investigates the adhesion of liquid alginate solutions to oesophageal tissue. This chapter describes the development of a suitable method to characterise the extent and duration of alginate adhesion to this tissue surface. Previous techniques used in this type of assessment are discussed and the development of the technique used is also detailed.
4.2 TECHNIQUES USED TO ASSESS BIOADHESION

Generally bioadhesive drug delivery systems have been formulated as solid compacts, flexible films, gels and visco-elastic semi-solids (Ahuja et al, 1997). Therefore many of the tests involved in the characterisation of such materials are not suited to the assessment of liquid formulations. Force of detachment tests are simple to perform and are a popular means of assessing the strength of the interaction between a bioadhesive formulation and a biological substrate. Rheological techniques are also a common tool used to probe the interaction between a bioadhesive formulation and a mucus substrate.

One of the first methods used to assess bioadhesion was developed by Park & Robinson (1984). This method used cultured epithelial cells that had been fluorescently labelled. A polymeric suspension was added to the cells and the change in fluorescence was indicative of the extent of interaction between the polymeric formulation and the epithelial cells. This technique is applicable to liquid formulations, however, disadvantages include the process involving a great deal of preparation plus the labelling of epithelial cells may greatly affect their inherent surface properties, thus the relevance of this model is limited.

Tensile strength measurements are a widely used method to measure the strength of the adhesive interaction. Such tests, developed by Smart et al (1984), measure the forces involved in pulling apart a biological substrate and an adhesive formulation. Both materials involved in the adhesive process must be gripped in some manner for an effective measurement to be obtained. Liquid formulations cannot be gripped, thus this test is unsuitable here. A second disadvantage of such a test includes the point of detachment. To effectively measure the strength of the adhesive bond it is this bond that should be broken in the test. However, generally it is the weakest interface that separates. This interfacial associative strength may be assessed but the relevance of such data is dubious.

There are only limited numbers of papers that investigate the bioadhesive properties of liquid-like formulations; these include papers that discuss the in situ gelling of suppositories (Kim et al, 1998) and those that discuss prolonging drug delivery to the
eye (Cohen et al, 1997; Davies et al, 1991). An ideal method to assess the retention of an alginate dose on oesophageal tissue requires:

- A suitable substrate
- An accurate method to assess the amount of the dose that adheres to the substrate
- Physiological similarity to the real situation
- A means of measuring the duration of the adhesion
- Minimalisation of the errors involved in measuring the adhesion of a dose

Many models were reviewed and investigated in order to reproduce an optimum method for measuring the adhesion of an alginate solution to oesophageal tissue. Teng & Ho (1987) used a method that investigated the retention of polymer coated latex beads on intestinal mucus; this method is alternatively termed the “falling film” method. Within this method coated beads are dispensed onto the intestine substrate then washed with an appropriate medium. The number of particles dispensed was known and those washed off were calculated using a Coulter counter. A similar method was used by Rao & Buri (1989), although they used glass beads and analysed the percentage of beads recovered by gravimetric analysis of the collected material. The falling film method, as described, has proved to be a useful means of assessing the strength and duration of adhesion of polymer coated particles and/or microparticles (Ascentiis et al, 1995).

Other methods that specifically target the oesophagus were also explored to assess their potential as a valid method for this study. These methods included an everted rat oesophagus model used to determine the retention of a sucralfate suspension (Dobrozsi et al, 1999). This is a novel method that uses everted rat oesophageal tissue that is immersed into a solution of the relevant polymer. This method is an adaptation of the tensile strength methods whereby the method has been adapted for use with liquid formulations. The initial mass of polymer retained on the tissue was assessed gravimetrically. The tissue was then washed in an appropriate medium and the mass retained at set time points was measured. This model has been shown to assess the in vitro potential of oesophageal mucoadhesive viscous liquids. One
disadvantage of this technique is that the method would be difficult to replicate using porcine oesophageal tissue due to the difference in size between the two tissue substrates. The advantages of porcine oesophageal tissue include its histological similarity to human oesophageal tissue and also the low expense and easy availability of this material.

A second method considered was a continuous-flow adhesion cell developed by LeRay et al (1999). A schematic representation of the apparatus used is shown in Figure 4.1. The method consisted of a glass tube with an opening at either end. The substrate was placed inside the tube and the bioadhesive formulation dispensed into the upper end of the tube. The substrate was washed with an appropriate medium and the effluent collected. The formulation was associated with a dye; this enabled colourimetric analysis of the effluent to determine the amount of the formulation removed from the biological substrate. Two substrates were compared; inert polymer tubing and ex vivo intestinal tissue.

![Figure 4.1. The continuous-flow adhesion cell apparatus used by LeRay et al (1999)](Image)

Another study that investigated adhesion onto the oesophagus used magnetic granules coated with a bioadhesive polymer to localise the formulation within the oesophagus (Nagano et al, 1997). These particles were administered then localised using a magnet. During the period for which the particles were held in contact with the mucosal surface the bioadhesive polymeric material hydrated and adhered to the tissue substrate. This type of formulation has applications in the treatment of
oesophageal cancer whereby side effects may be minimised due to localisation of the drug therapy.

The method used to assess alginate retention on oesophageal tissue was based on a combination of the falling film method, the everted rat oesophagus and the continuous flow adhesion model. In developing a method for use within this study the first consideration was in producing a method that was similar to the real physiological situation. The substrate used was porcine oesophageal tissue; this was prepared as described in Chapter 2. The method required an alginate dose to be dispensed onto oesophageal tissue then washed with a suitable medium that mimicked saliva flow. The percentage of the dose remaining on and washed off the tissue was calculated as a means of assessing the retention of an alginate dose. The final method used was developed by Banning (1999). Further details about the technique and the apparatus used are provided in the next section.

The aim of this project was to characterise the adhesion of an alginate solution on oesophageal tissue; in this case the role of the bioadhesive agent is in protection of the oesophagus and limiting the damage caused to this organ by the reflux of the stomach contents. The experimental work was divided into three sections; method optimisation, characterisation of the adhesion of an alginate solution on oesophageal tissue and using the adhesive layer as a means of supporting model drug particles.

### 4.3 Technique Used

The method used has been described previously (Banning et al, 1998; Batchelor et al, 1999) although an outline of the technique is provided below. The apparatus involved is shown in Figure 4.2.

This technique relies on the alginate solution being fluorescently labelled as the results were determined using a fluorimetric analysis technique. The methods involved in labelling the alginate were described in detail in Chapter 2. The alginate dose was dispensed onto oesophageal tissue and the mounting platform was inclined.
A peristaltic pump provided an appropriate washing medium that flowed over the tissue surface and was collected into a vial along with any of the alginate dose.

![Diagram of peristaltic pump and tissue setup](image)

**Figure 4.2.** The apparatus is shown from above (a) and in profile (b). The apparatus was used within a temperature and humidity controlled environment.

Fluorimetric analysis of the collected material allowed determination of the amount of the dose collected. By fixing the time points a profile of the amount of the dose collected from and the amount remaining on the tissue was prepared. The exact details of the technique are provided in the method section of this chapter.

### 4.3.1 Apparatus and instrumentation

The retention model used was constructed in-house and although the basic apparatus is shown in Figure 4.2 the finer details will be covered in this section. Within the study two sets of apparatus were used in order to perform the full study. Evaluations were made between the two sets of apparatus to ensure that the results given were directly comparable. This data is shown in Appendix II. The apparatus consisted of a controlled temperature and humidity cabinet, a mounting platform to support the tissue substrate, a peristaltic pump to deliver an appropriate medium to the tissue, collector vials and a fluorescence spectrometer to analyse the alginate content of the collected material.
4.3.1.1 Controlled temperature and humidity cabinet

The main apparatus used was a temperature and humidity controlled cabinet within which the study was performed. During the project two different cabinets were employed, initially an adapted Gallenkamp industrial humidity cabinet model BR185H was used. This monitored the temperature and humidity by means of a dry and wet bulb. The dry bulb was used to monitor the temperature and the difference between the temperature of the dry and wet bulb was adjusted using hygrometric tables to produce the required humidity. A second cabinet was used in later experiments; this is shown diagrammatically in Figure 4.3.

![Diagram of apparatus](image)

**Figure 4.3.** The second set of apparatus used to assess the retention of an alginate solution on oesophageal tissue

This second cabinet was simpler and was constructed from Perspex®. A ventilated Perspex® sheet was placed between the cabinet and the Gallenkamp 1H-350 waterbath maintained at 37 °C. This ensured that the cabinet temperature was kept at 37 °C and the humidity was held at a level greater than 90% RH. Comparative experiments were performed to ensure that the results obtained were not affected by...
the change in apparatus used. The results can be seen in Appendix II. A thermometer (BDH Commercial Grade Thermometer) and hygrometer (BDH Hair Hygrometer) were placed inside this cabinet to ensure that the temperature and humidity could be monitored throughout experimental procedures.

The rationale behind the temperature and humidity controlled cabinet was to mimic the conditions found in vivo, the temperature within the oesophageal cavity is 37 °C and the humidity is assumed to be close to 100 % RH. Sealed glove access in both cases allowed experimental manipulation to occur without altering the environmental conditions.

4.3.1.2 Mounting Platform

The mounting platform was a specially constructed piece of Perspex®, the dimensions of which were 130 x 57 mm. A section was taken from this Perspex® mount that measured 12 x 100 mm, it was into this groove that the tissue section was positioned. This is shown schematically in Figure 4.4.

![Figure 4.4. Schematic representation of the mounting platform](image)
The mounting platform was held in place by a clamp that could be adjusted to produce different slope angles. This was a key feature in the method development process. A wire loop was attached to the upper end of this mounting platform. The washing medium supplied by the peristaltic pump was maintained in position via the use of this loop.

4.3.1.3 Peristaltic Pump

The peristaltic pump used as part of this apparatus was a Watson Marlow model 202 pump. This pump allowed the flow rate of the washing medium to be controlled accurately. The tubing used in conjunction with the pump was split into four separate tubes to evenly distribute the washing material over the surface of the tissue section. This was illustrated in Figure 4.4. The material was split in an attempt to more accurately mimic saliva flow over oesophageal tissue in vivo. Splitting the delivery system also aided in providing an even flow of the washing medium over the tissue surface.

4.3.1.4 Fluorescence Spectrometer

Two different fluorescence spectrometers were used to analyse the collected fractions within the project. Although different instruments were used the principle of their operation is similar.

Molecular fluorescence is the optical emission from molecules that have been excited to higher energy levels by absorption of electromagnetic radiation. A fluorescent dye has a particular wavelength of electromagnetic radiation that will excite the molecules. Excited molecules emit light of a different wavelength to the initial radiation. The emitted light was detected at a perpendicular angle to the excitation light. This means that when the material is exposed to a specific radiation only the fluorescent molecules will be detected and non-fluorescent molecules will not contribute to the reading. This provides a clear advantage of fluorescence spectroscopy as a means of analysis. A typical fluorimeter contains an excitation source, a sample cell and a fluorescence detector. In both instances a xenon lamp was used as the excitation source and a quartz crystal 10 mm cuvette was used as the
sample cell. The degree of fluorescence measured is dependant on the strength of the excitation source; both fluorimeters allowed the strength of this source to be adjusted by means of slits. Narrowing the slits eliminates some degree of the excitation source thus the sample reading can be adjusted.

The initial spectrometer used was a Perkin Elmer Spectrophotometer PE-204 coupled to a Perkin Elmer 150 xenon power supply and PE-56 recorder. This instrument read a range of samples between an emission intensity of 0-100. Prior to sample measurement, a calibration was performed, the fluorescence value of the weakest sample must be detectable within the calibration range. 1% of the delivered dose was used as the 100 % calibrant. This concentration was chosen to ensure that all the samples collected could be measured within the calibrated range. Calibrants were prepared at each interval of 10 between 0 and 100 %. This instrument required manual manipulation to ensure that the 100 % calibrant solution read 100 on the display. This was performed for each analysis performed. Samples collected that had a high fluorescence value were diluted to ensure that the fluorescence was measured within the calibrated range. A disadvantage associated with this technique lies in this dilution process. 1 % of the original dose was set as the maximum recordable reading using the designated instrument. Any error in the measurement of a given sample corresponds to a large associated error in the data manipulation process.

The second spectrometer used was a Perkin Elmer PE - LS-5 Luminescence Spectrometer with a xenon power supply. The methodology varied slightly with this instrument in that the emission intensity was measured directly and the value found for the 100 % calibrant solution did not necessarily read 100 on the output device. However, the output could read between 0 and 999.9 so the slits were adjusted so that the 100 % calibrant solution read approximately 700 on the scale. This procedure ensured that the lowest concentration samples would be read within the calibration range. Calibration curves for both instruments can be seen in Figure 4.5.
Figure 4.5. Calibration curves for (a) the Perkin Elmer PE 204 spectrometer and (b) the PE LS-5 spectrometer

Both calibration curves shown in Figure 4.5 were produced from at least four repeats in order to maximise the precision of this data. Both instruments produced successful linear calibration curves ($R^2 > 0.9$) and were easily able to collect precise data. Further details about the calibration procedure are given in the next section of this chapter.
4.3.2 Experimental procedure

The porcine oesophageal tissue, prepared as described in Chapter 2, was trimmed to fit a groove 12 mm by 60 mm cut into the mounting platform. It was then placed epithelial side upwards as shown in the apparatus pictured in Figure 4.4. The mounting platform was set to the horizontal and a measured alginate dose, prepared as described in Chapter 2 was delivered to the tissue. The tissue was left at the horizontal for a set time, termed the equilibration time. This time period facilitated even distribution of the dose before the tissue was set to a specified angle. The washing medium was set up to flow evenly over the tissue-alginate surface and the effluent was collected from the surface at designated time intervals. Each experiment was performed a minimum of six times and an average value was produced. Measurement of the effluent using a fluorescence spectrophotometer allowed calculation of the amount of the measured dose washed off the tissue at any given time point. This calculation was performed via calibration curves.

4.3.2.1 Calibration curve

Calibration curves for both the covalently labelled alginate and the alginate-fluorescein solution were prepared. In each case, a 2 % w/v solution of alginate was prepared. 1 ml of this solution was taken and made up to 100 ml in a volumetric flask by the addition of deionised water. This solution was used as the maximum emission intensity value as during the experimental process the alginate dose was greatly diluted by the washing medium. Thus the 100 % calibrant solution contained 0.2 mg/ml of alginate. Subsequent concentrations were prepared by dilution of this stock solution. 10 calibrant solutions in total were prepared. Figure 4.6 shows a typical calibration curve for the covalently labelled alginate. Fresh calibration curves were prepared for each experiment as differences may occur between standards for each procedure and the nature of the dose may vary between experiments.
Figure 4.6. Calibration curve prepared using covalently labelled fluorescent alginate

Figure 4.6 shows a calibration curve produced from the average of 6 repeats of a calibration experiment to assess the emission intensity of alginate solutions of known concentration. The equation of the line of best fit is shown, this is used to calculate the concentration of alginate from the emission intensity within the experimental procedure.

4.3.2.2 Calculations

The concentration of alginate present in the effluent samples collected was calculated via the calibration curve; the calculations involved in this process are detailed below. The volume of the effluent collected must be considered when the fluorescence concentration is correlated to the alginate concentration as the calibration curves examine the fluorescence present in milligrams per millilitre. In each fraction there was likely to be more than one millilitre collected thus the total alginate washed off the tissue must be accounted for. The emission intensity of the collected fraction was noted; this was then multiplied by the volume of the collected fraction. The volume was measured by means of a pre-calibrated 10 ml graduated glass pipette. This pipette was used to transfer the fraction from the collection vial to the cuvette. This method of transfer allowed the volume of each fraction to be recorded.
Emission Intensity (EI)* Volume = Emission Intensity of the total fraction

Equation 4.1

The next manipulation was to determine how the amount present in the fraction was related to the dose placed on the tissue surface. The calibration curve was used for this purpose. The line of best fit was used to determine the emission intensity of 1% of the dose. As 1 ml of the dose was diluted into a 100 ml volumetric flask, the 100% calibration solution represented 1% of the dose. Thus according to the line of best fit when \( x = 100 \), the emission intensity, relates to that of 1% of the dose. 1% of a 2% w/v alginate solution contained 0.2 mg/ml of alginate.

\[
\text{If } x = 100 \% \text{ then } y = m(100) + c
\]

Equation 4.2

\[
\text{or if } x = 0.2 \text{ mg/ml then } y = m(0.2) + c
\]

Equation 4.2 allows the emission intensity of 1% of the dose to be calculated as:

\[
y = 1 \% \text{ of the delivered dose}
\]

Thus the emission intensity found for the total fraction was divided by that found for 1% and the percent of the dose removed at each time point was calculated. Equations 4.1 and 4.2 are combined to determine the amount present in the fraction collected as a percentage of the total dose.

\[
\frac{\text{Emission intensity of total fraction}}{\text{Emission intensity of 1% of the dose}} = \text{amount collected as a } \%\text{ of the dose}
\]

Equation 4.3

The procedure was performed for each fraction and the cumulative percentage removed was determined at each time point. An example calculation is outlined below.
An example calculation is performed according to the calibration shown in Figure 4.6. The emission intensity for the fraction was found to be 525 and the volume was 2.7 ml.

The emission intensity for the total fraction would be:

$$525 \times 2.7 = 1417.5$$

Equation 4.1

According to the calibration curve the line of best fit has the equation

$$y = 3390.3x - 29.658$$

As the dose was diluted by a factor of 100 a 1% dose would have an alginate concentration of 0.2 mg/ml and thus the emission intensity would read;

$$y = 3390.3(0.2) - 29.658 = 648.402$$

Equation 4.2

The percentage of the dose removed in this fraction was:

$$\frac{1417.5}{648.402} = 2.9\%$$

Equation 4.3

This calculation was performed for each fraction, which allowed the cumulative percent of the dose removed to be determined.

4.4 AIMS OF THE STUDY

This study investigated the retention profile and duration of retention of an alginate dose on porcine oesophageal tissue. Initial experiments were performed as part of the method development. The system used was an *in vitro* model and the conditions were maintained as close to physiological conditions as was feasible. This increased the relevance of the model. The method optimisation study investigated experimental parameters to observe the effects of aspects of the experimental design. Standardisation tests were also performed to assure the viability of the method used.
These tests included control experiments to demonstrate that the effects observed were true effects. Blank samples were run on the oesophageal tissue and inert substrates were used instead of oesophageal tissue in order to validate the model. Finally, various experimental parameters were investigated using the developed and validated method. These tests included:

- Size of alginate dose
- Concentration of alginate dose
- Duration of retention; the retention was monitored for 60 minutes
- Washing medium; using artificial saliva to determine the effect on the retention of the alginate dose
- Nature of the alginate used as the dose; a range of alginates were investigated to note their retentive properties
- Presence of the unstrirred water layer; the unstrirred water layer was removed and the adhesion profile monitored.
- Retention of fluorescently labelled beads that acted as model drug particles.

4.5 RESULTS AND DISCUSSION

The experiments were performed under a set of standardised conditions. These included a set dose size of 1 ml and an alginate concentration of 2 % w/v. Retention was assessed at 3 minute intervals up to 30 minutes. Sodium alginate, LF120, associated with disodium fluorescein was used, the preparation of this material was described in Chapter 2. Deionised water was used as the washing medium and it was set to flow over the tissue surface at a rate of 1 ml / minute. The temperature was set to 37 °C and the humidity was maintained at a level greater than 90 % RH. Initial experiments performed were those that optimised the method.

4.5.1 Method Optimisation Experiments

Initial experiments were performed in order to optimise the method. These experiments investigated phenomena associated with the set up of the apparatus.
4.5.1.1 Angle of inclination

The human oesophagus is usually vertically orientated, thus in an ideal model the tissue should be set to an almost vertical position. In reality this would make the experimental procedure very difficult, the shallower the angle of inclination the easier it is to ensure that the entire alginate dose washed off the tissue was collected in the designated vial. A range of angles was investigated to determine the effect of the angle of inclination on the experimental parameters; the results are shown in Figure 4.7.

![Graph showing the effect of the angle of inclination on the retention of an alginate dose](image)

**Figure 4.7.** The effect of the angle of inclination on the retention of an alginate dose

The results shown in Figure 4.7 suggest that there was no difference in the adhesion of alginate to oesophageal tissue when the tissue was inclined to various angles between 45° and 75° to the vertical. Neither the angle of inclination nor the time point investigated demonstrated a significant difference in the retention of the alginate dose ($p > 0.05$ in all cases). An angle of 60° was chosen for subsequent experiments as this gave reproducible results and experiments that were simple to perform.
4.5.1.2 Flow rate

Many references are available that quote the flow rate of unstimulated human saliva in healthy control subjects. Wang et al (1998) reported a flow rate of 0.33 ml/min, alternatively Wolff & Kleinberg (1998) reported a flow rate of 0.39 ml/min. However, a maximum flow rate of 10 ml/minute of stimulated saliva was reported by Dreizen et al (1968). The cuvette used in conjunction with the spectrometer required at least 2 ml volume in order to record an emission intensity reading. Due to the already large errors associated with this measurement system as discussed in section 4.3.1.4 of this chapter, it was desirable to use the collected fraction undiluted. Thus, a collected volume of 3 ml was set as a target. For example a flow rate of 1 ml/minute allows fractions to be collected every 3 minutes. A physiologically relevant flow rate was desirable to add merit to the model as developed. However, a greater flow rate would allow the fluorescence to be measured at more frequent time intervals and thus a clearer picture of the adhesion profile of an alginate dose to porcine œsophageal tissue may be visualised. A flow rate of 0.4 ml/minute was compared to a faster rate of 1 ml/minute. The results are shown in Figure 4.8.

![Figure 4.8. The effect of flow rate on the retention of an alginate dose](image)

The collection points were different for the two flow rates as, as stated previously, a set volume was required to fill the cuvette used in conjunction with the fluorescence spectrophotometer. The adhesion profile of alginate to œsophageal tissue was not affected within the flow rates investigated. Statistical analysis showed no significant difference (p > 0.05 in all cases) in the retention of the alginate dose according to the
flow rate. No significant differences were observed in the alginate retention at all three time point investigated (p > 0.05 in all cases) A flow rate of 1 ml/minute was selected for future work as it allowed more frequent analysis of the adhesion of the alginate on the tissue surface. This rate allowed fractions to be collected every 3 minutes for a 30 minute period. Although this flow rate did not provide the most physiologically real situation, it allowed a greater number of collection intervals to be monitored and the decision was justified in this manner.

4.5.1.3 Equilibration time

The length of time for which the alginate dose was held on the tissue surface prior to inclining the tissue to the desired angle was varied; this time period may be termed the equilibration time as it allowed the alginate dose to rest and first form a layer on the œsophageal mucosa. Figure 4.9 compares a 60 second with a 300 second equilibration time.

![Graph showing equilibration time vs. retention of alginate dose](image)

**Figure 4.9.** The effect of equilibration time on the retention of an alginate dose

No significant differences were observed between the retention of the alginate dose according to the equilibration time period (p > 0.05 in all cases). The time period investigated also showed no significant differences (p > 0.05) in the retention of the alginate dose. It is difficult to estimate how long the alginate would remain in intimate contact with the œsophagus before being disturbed. Leaving the alginate in contact with the epithelial layer for a greater length of time may have aided the
interaction by increasing the potential of specific interactions occurring. However, as a 300 second equilibration time seemed physiologically unlikely, a 60 second time period was selected.

4.5.1.4 Humidity effects

The humidity of the experiments was controlled as described earlier. As the oesophagus is a wet environment, the humidity is expected to be approximately 100% relative humidity (RH). An experiment was performed to assess the difference in adhesion at a humidity greater than 90% RH compared to 60% RH. Figure 4.10 shows the results of this investigation. Statistical analysis showed no significant differences in the results obtained according to the humidity (p > 0.05).

![Chart showing the effects of humidity on adhesion of alginate to porcine oesophageal tissue](image)

**Figure 4.10.** The effects of humidity on adhesion of alginate to porcine oesophageal tissue

The humidity did not affect the adhesion of alginate to oesophageal tissue yet it seemed more appropriate to conduct future experiments at a humidity greater than 90% RH as this is, physiologically, a more real situation. The initial cabinet used as part of the apparatus had very accurate humidity control, whereas the second piece of apparatus used could control the humidity only as being greater than 90% RH throughout the experimental procedure. Appendix II highlights the retention of an alginate dose using the two different sets of apparatus, no difference was seen in the
retention of the alginate dose. Again no significant differences were observed in the retention of an alginate dose according to the time points investigated (p > 0.05 in all cases).

4.5.1.5 Choice of label

The effect of the technique used to label the alginate was investigated. The alginate-fluorescein solution was far easier to prepare compared to the covalently labelled alginate, thus it was desirable to use the alginate-fluorescein solution in the experimental procedure. Figure 4.11 compares the adhesion of the alginate solution to esophageal tissue according to the labelling technique used. The covalently labelled alginate was expected to give a more realistic profile as the label was unable to disassociate and potentially produce unreliable results.

![Graph comparing retention of alginate dose](image)

**Figure 4.11.** Comparison of the retention of an alginate dose using different labelling techniques

The adhesion profile of the covalently labelled and alginate-fluorescein solutions are somewhat similar. Initially the profiles varied, although after 10 minutes the profiles were almost identical. Statistical analysis performed on the retention of the alginate dose according to the labelling method used showed significant difference at the 3-minute time point (p-value < 0.05). At 15 and 30 minutes the difference in the retention of the two doses was not significant, (p > 0.05). This initial variation between the two labelling techniques may be due to saturation of the sodium
fluorescein label. The sodium fluorescein used in the initial dose was at a high concentration to ensure that the last fractions collected were measurable within the calibration range. Thus the initial fractions may be saturated with the label and the results may be affected. Alternatively, the slight difference in viscosity between the two labelled alginates may lead to the variation in the retention profile of the differently labelled alginates. The covalently labelled alginate has a slightly higher viscosity (as noted in Chapter 2); this factor could limit the ability of the washing medium to remove the applied dose. The viscosities of the two alginates were assessed at 37 °C at a shear rate of 10 s⁻¹. The fluorescein-alginate solution exhibited a viscosity of 0.51 (± 0.02) Pa.s whereas the covalently labelled alginate exhibited a viscosity of 0.75 (± 0.03) Pa.s. In further experimental procedures the sodium fluorescein-alginate was used preferentially. This is due to the simple procedures involved in the preparation and use of this material as compared to the covalently labelled alginate.

4.5.2 Standardisation experiments

The experiments performed thus far allowed the method to be optimised. The angle of inclination was set at 60° to the vertical and the flow rate at 1 ml per minute. The equilibration time was set at 60 seconds and the humidity at greater than 90 % RH. The alginate associated with sodium fluorescein is used preferentially to the covalently labelled alginate, as this preparation procedure was simpler. Standardisation tests were then performed to establish the validity of the method.

4.5.2.1 Inherent fluorescence of the œsophageal tissue

This experiment determined the background fluorescence of œsophageal tissue with deionised water as a washing medium. The spectrometer was set up for a standard experimental procedure. Neither alginate nor fluorescent dye was applied to the tissue surface; the tissue was washed at a flow rate of 1 ml/ minute with deionised water and the effluent collected. Fractions were collected every 3 minutes for a 30 minute period and analysed using the fluorescence spectrometer. The results showed that the none of the collected fractions possessed an emission intensity greater than the blank value determined in a standard calibration procedure. This
proves that the œsophageal tissue does not contribute any background fluorescence and that the fluorescence measured is derived purely from the labelled dose.

4.5.2.2 *Inherent fluorescence of unlabelled alginate dose*

This experiment used unlabelled alginate as a control in order to assess the background fluorescence of the alginate dose. As with the above result, no fluorescence was detected as all fractions analysed showed emission intensity values equal or lower than the blank. This experiment demonstrated that the alginate dose does not contribute to the fluorescence measured.

4.5.2.3 *Aqueous dose of sodium fluorescein*

This standardisation method used a solution of sodium fluorescein in water without any alginate component. This experiment acted to ensure that the alginate dose did remain associated with the sodium fluorescein. It was expected that all the sodium fluorescein would be removed from the tissue surface rapidly as there was no bioadhesive component present. The results showed that at 3 minutes 98.65 % (± 6.8) of the dose had been removed and at 6 minutes 100.87 % (± 7.3) has been removed. After this time point all the values showed that the entire dose had been removed. This is shown in Figure 4.12.

![Figure 4.12. Comparison of the retention of an aqueous solution of sodium fluorescein as compared to an alginate-fluorescein solution](image_url)
Standard conditions as labelled on Figure 4.12 refers to a 1 ml dose of 2 % w/v LF120 alginate solution delivered to oesophageal tissue. This dose was labelled using sodium fluorescein and the difference in retention between this dose and an aqueous solution of sodium fluorescein were compared. Figure 4.12 shows the percentage of the original dose washed off the tissue rather than the percent retained on the tissue that has been shown previously. At 3, 15 and 30 minutes significant differences were observed in the retention of the doses applied. Within each series no significant difference was noted between the retention of the dose at 3, 15 and 30 minutes (p > 0.05 in all cases).

4.5.2.4 The retention of alginate on a polymeric substrate

Dialysis tubing (supplied by Sigma, UK) was used as an inert polymer substrate to assess the retention of an alginate dose. This control was used to indicate whether the alginate adhesion was a phenomenon associated with the oesophageal substrate rather than a rheological event. Figure 4.13 shows the retention of an alginate dose on inert tubing as compared to porcine oesophageal tissue.

![Comparison of retention](image)

*Figure 4.13. Comparison of the retention of an alginate dose on oesophageal tissue compared to inert polymer tubing*

This comparative figure shows that alginate was retained to a certain extent on inert polymer tubing for 15 minutes. Statistical analysis showed significant differences
between the biological tissue and the inert substrate at both 3 minutes and 30 minutes (p < 0.05). At 15 minutes the difference was not significant with the p-value calculated as being 0.44. However, the percentage of alginate retained on oesophageal tissue was greater at each time point and the duration of adhesion also appeared to be longer. Each series showed no significant differences in the retention of the dose at any of the time points investigated. Certain studies (LeRay et al, 1999) have shown that inert polymeric materials can act as substrates on which adhesion can be assessed. According to these results this substrate would not be suitable within this model for such an assessment. The results of this study also suggest that the alginate is adhering to the oesophageal tissue via specific linking rather than physical phenomena.

4.5.3 Characterisation of the adhesion of an alginate dose to oesophageal tissue

The optimised and validated method was used to assess a number of different situations. The size and concentration of the alginate dose was varied as well as a range of alginates being investigated. The retention of the range of alginates was examined and the physico-chemical properties of the alginates were related to the extent and duration of adhesion. The range of alginates investigated included LF120, SF120, LFIOL, LFR5/60, SF200 and H120L. Their properties were listed in Table 2.1 of Chapter 2.

4.5.3.1 Size of the Alginate Dose

The effect of applying a 2 ml dose of the alginate solution as compared to a 1 ml dose was investigated. The results are shown in Figure 4.14. These doses were applied to the same area of oesophageal tissue and their retention compared at 3, 15 and 30 minutes. No significant differences were observed in the retention of the different sized doses at 3, 15 and 30 minutes. No significant differences were noted in the retention of a 1 ml dose over the three time points investigated. The 2 ml dose showed a significant difference in the retention of the dose at 3 minutes compared to 30 minutes although no significant differences were observed between 3 and 15 minutes and 15 and 30 minutes.
Figure 4.14. Comparison of the effect of dose size on alginate adhesion

The difference in the values seen for the mean percentages of the alginate dose retained on the tissue are a phenomenon associated with the different sized doses. At the latter time points, the percentage of the 2 ml dose retained was approximately equal to half the percentage of the 1 ml dose. This phenomenon is an artefact of the different sized doses. The actual amount of alginate retained in each situation was similar. Approximately the same volume of alginate solution is maintained in contact with the designated area of exposed tissue. This occurrence is indicative of a specific interaction holding the alginate onto the mucosal surface rather than just a physical phenomenon.

4.5.3.2 Concentration of the alginate dose

Experiments performed on the different alginates included an investigation into the concentration effects of the alginate solution on the oesophageal tissue as well as the effect of using a range of different alginates. The results of the concentration differences are shown in Figure 4.15. Sodium alginate LF120 was made up in three solutions of different concentrations, 2, 3 and 5 % w/v. The adhesion profiles of these concentrations were investigated.
Figure 4.15. Effect of the concentration of the alginate solution on the retention of the dose

These results show that at 3 minutes significant differences were observed in the retention of all three doses of different concentration. No significant differences were observed between the doses at either 15 or 30 minutes. The 2% w/v dose showed no significant difference in its retention over the entire time period investigated. Both the 3% and 5% w/v doses exhibited significantly higher retention at 3 minutes compared to the later time points. These results show that the profiles of adhesion vary with concentration in the initial stages. Yet at 15 and 30 minutes the actual amount of alginate retained on the tissue substrate was similar in all three cases. These results are also represented over the entire time period to show the similarities in the retention profiles. Figure 4.16 shows these results.
Figure 4.16. Comparison of the retention of a 2, 3 and 5 % w/v dose of alginate solution on porcine oesophageal tissue

This graph highlights the differences found between the retention of the doses of different concentrations in the early stages of the experimental procedure. The percentage of the dose washed off during the entire 30 minute period was similar in all three cases. The prepared alginate solutions had different viscosity values as higher concentrations possess greater viscosity values. It may be that the higher viscosity alginates are retained on the oesophageal tissue to a greater extent in the initial parts of the study yet the extent of retention is less affected by the viscosity at later time points. This result corresponds to the earlier result comparing the retention of the alginate according to the labelling technique used. The higher viscosity, covalently labelled alginate was retained to a greater extent in the initial stages compared to the lower viscosity alginate-sodium fluorescein solution. It may be suggested that alginate retention is dependant upon viscosity in the initial stages of the time period investigated.

4.5.3.3 Duration of retention

The retention of the alginate dose on oesophageal tissue was investigated for a period of 60 minutes instead of the usual 30 to determine the duration of adhesion for time periods of one hour. The results are given in Figure 4.17.
Figure 4.17. Comparison of the retention of an alginate dose on oesophageal tissue for a period of 30 and 60 minutes

The graph clearly shows that the retention profiles were very similar for the initial 30 minutes in both situations and that after 60 minutes 17.9% of the original dose was still retained on the oesophageal tissue. No significant differences were observed in the retention of the two doses at all time points investigated up to 30 minutes ($p > 0.05$). The dose was retained for up to 60 minutes with very little loss occurring after 30 minutes. This result suggests that an adhesive system may be localised within the oesophagus for time periods of at least one hour.

4.5.3.4 Washing medium

As discussed in Chapter 2 the use of natural saliva in experimental procedures carries its own difficulties due to the inherent variability of this fluid. However, the adhesion of an alginate solution to the oesophagus may be greatly affected by the washing action of saliva. Previous experiments have used deionised water as a washing medium. The viability of the results collected so far were assessed by comparing the retention found using deionised water, natural saliva and two formulations of artificial saliva. Further information on the artificial salivas used and the rationale behind their inclusion can be found in Chapter 2. Figure 4.18 compares the retention of an alginate dose using the four different washing media.
Statistical analyses performed on these results showed that no significant differences were observed in the retention of the alginate dose at 3, 15 and 30 minutes according to the washing media used. Neither natural saliva, deionised water or artificial saliva II showed significant differences in the retention of the alginate dose at the three time points investigated. However, artificial saliva I showed significantly higher retention at 3 minutes compared to 30 minutes. This system did not show significant retention value differences between either 3 and 15 or 15 and 30 minutes.

It was far easier to use deionised water to perform studies due to its ready availability; hence, this medium was used throughout the remainder of the experimental procedures. Any differences seen in the retention of the dose using the different media may be attributed to the mucin content of the washing medium. If the adhesion profiles are ranked, greatest retention is observed with deionised water and artificial saliva and artificial saliva II exhibited the lowest retention. Ranking the media used according to mucin concentration shows; natural saliva > artificial saliva II > artificial saliva I > deionised water. The trend in mucin concentration was not related to the retention order and this result indicates that the mucin content of the washing medium over the range investigated does not influence the retention of an alginate dose.
4.5.3.5 Comparison of a range of alginates

The procedure as developed was used to investigate the retention of a range of alginates on oesophageal tissue. The properties of the range of alginates investigated were listed in Table 2.1 in Chapter 2. Figure 4.19 below shows the retention of a 1 ml, 2 %w/v dose of each alginate.

The retention of the alginates varied the most in the initial stages with the amount retained being greatest for those alginates with the highest viscosity, namely SF200 and H120L. The two alginates with very low viscosity, LFR5/60 and LF10L were retained the least both initially and throughout the 30 minute monitoring period. This is shown more clearly in Figure 4.20, this demonstrates that at 3 minutes the retention of the alginate dose was dependant upon the alginate used although at time points greater than 15 minutes the retention is less dependant upon the alginate used. Statistical analyses were performed on the retention of the range of alginates investigated and these results are discussed in fuller detail in conjunction with Tables 4.1 and 4.2.

Figure 4.19. Comparison of the retention of six different alginates on porcine oesophageal tissue
Figure 4.20. Comparison of the retention of a range of alginates at designated time points.

The alginates can be bracketed into groups of low, medium and high viscosity. LFR5/60 and LF10L are low viscosity alginates and they appear to be retained to a less extent than SF200 and H120L, high viscosity alginates. Statistical analysis was performed on the data collected and is shown and discussed in Table 4.1 later in this chapter. It can be suggested that the viscosity plays a role in the adhesion of alginate to porcine œsophageal tissue. Figure 4.21 compares the effect of viscosity on the retention of the alginate dose at both 3 and 30 minutes.

Figure 4.21. The effect of viscosity on the retention of an alginate dose.
The graph above shows that at 3 minutes the retention of the alginate dose is very dependant upon the viscosity of the alginate ($R^2 > 0.9$). At 30 minutes the retention did not appear to be linearly related to the viscosity of the alginate dose ($R^2 = 0.34$). This result corresponds with previous data collected when investigating the effect of the alginate concentration and the difference in the retention when using covalent alginate as compared to alginate-fluorescein solutions. In each of these situations higher viscosity alginates are retained for a greater length of time in the initial stages of the adhesion profile.

The alginates can also be grouped according to their monomeric make-up. LFR5/60 and SF200 are high G alginates whereas LF10L and H120L are low G alginates. The retention of the alginate according to the g fraction at both 3 and 30 minutes is shown in Figure 4.22.

![Figure 4.22. The effect of alginate G fraction on the retention of an alginate dose](image)

Figure 4.22. The effect of alginate G fraction on the retention of an alginate dose

Figure 4.22 above demonstrates that the retention of the alginate was not affected by the monomeric make up of the alginate moiety. At both 3 and 30 minutes the regression of the linear line of best fit was less than 0.1 indicating that there was no linear relationship between the retention of the alginate dose and the G fraction of the alginate.
Statistical analysis was performed on the entire alginate range at 3, 15 and 30 minutes. This analysis highlighted the differences in retention amongst the alginate range. A p-value of less than 0.05 indicated a significant difference in the retention of the two alginates considered. The analysis performed was an analysis of variance (ANOVA) as described in Chapter 2. The significance level chosen was 5 %. The results are shown in Table 4.1.

Table 4.1. p-values from statistical analysis of the retention of the alginate range on oesophageal tissue. (3 minutes, 15 minutes and 30 minutes).

<table>
<thead>
<tr>
<th></th>
<th>LF10L</th>
<th>SF120</th>
<th>LF120</th>
<th>SF200</th>
<th>H120L</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFR5/60</td>
<td>0.5860</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
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<tr>
<td></td>
<td>0.4992</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
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<tr>
<td></td>
<td>0.0539</td>
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<td>&lt; 0.05</td>
</tr>
<tr>
<td>LF10L</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
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<tr>
<td></td>
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<td>&lt; 0.05</td>
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<td>SF120</td>
<td>X</td>
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<td></td>
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<tr>
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<td></td>
<td>0.5335</td>
<td>0.3997</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF120</td>
<td>&lt; 0.05</td>
<td>X</td>
<td>0.1529</td>
<td>0.1118</td>
<td>0.2970</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>0.8225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF200</td>
<td></td>
<td>X</td>
<td></td>
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<td>0.8690</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.2094</td>
</tr>
</tbody>
</table>

The p-values were calculated using the statistical procedure described in Chapter 2. Table 4.1 shows the differences observed in the retention of the alginates at the three time points investigated. A p value of less than 0.05 indicated a significant difference between the retention of the two alginates under comparison. It can be seen that the retention of LFR5/60 was significantly different to all the other alginates with the exception of LF10L. Both LFR5/60 and LF10L are low viscosity
alginites. LF10L also displays a significant difference in its retention compared to the remainder of the alginate range. This result indicates the relationship discussed previously linking alginate viscosity to retention. Both LF120 and SF120 are mid range viscosity alginates. Significant differences were noted at 3 minutes between these alginates and the two high viscosity alginates although at later time points no significant differences were noted. These results agree with those displayed in Figure 4.21 linking alginate viscosity at 3 minutes with reduced retention. SF200 and H120L, the two high viscosity alginates showed no significant differences in their retention at all time points investigated.

Table 4.2 compares the retention of each alginate at the 3 different time points investigated. Y or N indicate the presence or absence of a significant difference in the retention of the dose at the time points indicated.

Table 4.2. Comparison of the retention profile of a range of alginates on oesophageal tissue

<table>
<thead>
<tr>
<th>Alginate</th>
<th>Time difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 - 15 minutes</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>N</td>
</tr>
<tr>
<td>LF10L</td>
<td>N</td>
</tr>
<tr>
<td>SF120</td>
<td>N</td>
</tr>
<tr>
<td>LF120</td>
<td>N</td>
</tr>
<tr>
<td>SF200</td>
<td>Y</td>
</tr>
<tr>
<td>H120L</td>
<td>Y</td>
</tr>
</tbody>
</table>

At the time points investigated both high viscosity alginates, H120L and SF200 showed significantly higher retention at 3 minutes compared to both 15 and 30 minutes. This may be an artefact of the relationship observed between alginate viscosity and the retention of an alginate dose in the initial stages shown in Figure 4.21. The only other significant difference observed in retention in relation to time
was with LF10L where the retention at 30 minutes was significantly lower at 30 minutes compared to 3 minutes.

4.5.3.6 Removal of the unstirred water layer

Chapter 3 investigated the composition of the unstirred water layer resident on oesophageal tissue. The importance of this layer in the retention of an alginate dose was examined in this study. Alginate has been shown to adhere to oesophageal tissue although the mechanism for this adhesion has not been elucidated. This experiment was performed to determine whether the adhesion was associated with the unstirred water layer resident on oesophageal tissue. The oesophageal tissue was scraped to remove the unstirred layer, this procedure was described in Chapter 2. The results of the adhesion studies on scraped and unscraped tissue are shown in Figure 4.23.

![Figure 4.23. Comparison of the adhesion of an alginate solution on scraped and unscraped oesophageal tissue](image)

Statistical analyses were performed to compare the retention of an alginate dose on natural (unscraped) and scraped tissue. No significant differences were observed in the retention of the dose at the initial three time points ($p > 0.05$). However, from 12 minutes to 30 minutes the retention of the dose on natural tissue was significantly higher than the retention values noted for scraped tissue.
The scraped tissue showed a very reduced adhesion profile with 100% of the dose being washed off within 15 minutes. The result demonstrated clearly the important role of the unstirred water layer in the adhesion of an alginate solution to oesophageal tissue. Previous studies performed comparing different washing media suggested that the mucin content was not relevant in the adhesion of an alginate dose. Therefore it may not be the mucin within this layer that holds the key to the difference in the adhesion profile of alginate to scraped and unscraped oesophageal tissue. However, scraping the tissue reduces the tissue surface roughness as seen in Figure 3.3 in Chapter 3 and it may be this physical difference in the tissue surface properties that affected the adhesion of the alginate solution. The inert polymer substrate investigated in section 4.5.2.4 also had a very smooth surface and the adhesion again was reduced. Figure 4.24 compares the adhesion of the alginate solution on unscraped and scraped porcine tissue as well as on dialysis tubing.

![Comparison of the alginate retention on various substrates](image)

**Figure 4.24.** Comparison of the alginate retention on various substrates

This figure illustrates the difference in the retention of the alginate dose when applied to either natural porcine oesophageal tissue, scraped tissue or an inert substrate. The natural tissue showed a retention profile that was different to both alternative substrates. However, the two alternative substrates exhibited similar adhesion profiles. This result adds strength to the theory that surface roughness may be a contributory factor to the adhesion of an alginate solution using the model described.
4.5.4 Investigation into the ability of the adhesive layer to support model drug particles

As demonstrated, alginate may adhere to oesophageal tissue for at least 60 minutes. This provides a mechanism through which drugs may be delivered directly to the oesophagus, or alternatively as a means of supporting particular sodium bicarbonate which could provide a secondary means of neutralising refluxed acid.

To investigate the feasibility of using this layer as a means of supporting particles fluorescently labelled latex beads were used as model particles. The fluorescent nature of the beads allowed their retention to be measured directly using a fluorescence assay technique. Fluorescent beads of different sizes and different surface charges were investigated. The properties of the beads used within this study are outlined in Table 4.3.

Table 4.3. Comparison of the beads used within the study

<table>
<thead>
<tr>
<th>Modification</th>
<th>Supplier</th>
<th>Diameter (μm)</th>
<th>Fluorescence</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Polysciences</td>
<td>0.05</td>
<td>Yellow-Green</td>
<td>458</td>
<td>540</td>
</tr>
<tr>
<td>None</td>
<td>Polysciences</td>
<td>0.5</td>
<td>Yellow-Green</td>
<td>458</td>
<td>540</td>
</tr>
<tr>
<td>None</td>
<td>Polysciences</td>
<td>2.0</td>
<td>Yellow-Green</td>
<td>458</td>
<td>540</td>
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<tr>
<td>Amine</td>
<td>Sigma</td>
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<td>489</td>
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<tr>
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<td>Yellow-Green</td>
<td>276</td>
<td>569</td>
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</tbody>
</table>

The three different sized beads were used to assess the ability of the alginate layer to support beads of different sizes. The amine, carboxylate and sulfite modified beads were used to assess the retention of a model drug particle with different surface charges.
4.5.4.1 Retention of particles of different sizes

Two drops of the fluorescent bead suspension, as supplied, were added to the prepared alginate (LF120) solution. This solution was stirred vigorously to ensure even distribution of the beads. Calibration curves were produced as described previously.

The three different sized beads were compared to discover the effect of the bead size on the retention of the model drug particles. The results are shown in Figure 4.25.

![Figure 4.25](image)

**Figure 4.25.** Comparison of bead size on the retention of an alginate dose

This graph shows the adhesion characteristics of fluorescently labelled beads within an adhesive alginate layer on oesophageal tissue. The retention profiles were similar and statistical analysis demonstrated that at each time point investigated no significant differences were observed in the retention of the beads according to their size (p > 0.05). In each case the retention at 3 minutes was significantly lower than at 15 and 30 minutes (p < 0.05). No significant differences were noted between the retention at 15 and 30 minutes. These results suggest that an adhesive alginate layer is able to support beads of diameter 0.05 - 2 μm for up to 30 minutes. The retention of the different sized beads was also investigated in an aqueous delivery system. At 3 minutes 86.3 (± 2.5), 89.5 (± 3.0) and 88.8 (± 2.4) % of the original dose had been collected for the 0.05, 0.5 and 2.0 μm beads respectively. In each case the entire
dose had been collected after 6 minutes of washing. These results were significantly different ($p < 0.05$) to those obtained for the retention of the beads suspended in alginate.

4.5.4.2 Retention of particles of different surface charge

The surface properties of a model drug may have a significant effect on its retention within an adhesive alginate layer. As alginate is an anionic polymer it may be expected that cationic drug particles would be supported to a greater extent. Three differently modified beads were used to test this theory. The results can be seen in Figure 4.26.

Figure 4.26. Comparison of the retention of amine, carboxylate and sulfite modified beads suspended within a 2% LF120 alginate solution

These results showed differences in the retention of the beads according to their surface charge. There was no discernible trend in their retention profiles. Statistical analysis was performed at 3, 15 and 30 minutes to determine the difference between the retention of the beads according to their charge. Significant differences were observed ($P < 0.05$) at 3 and 30 minutes although at 15 minutes no significant difference was observed. The profile observed for sulfite modified beads was very different to previous profiles examining alginate adhesion. Both the amine and carboxylate modified beads showed retention profile patterns similar to previous results investigating the adhesion of alginate to oesophageal tissue. In order to
validate the method a 1 ml dose of each aqueous suspension of the beads was also added to the oesophageal tissue alone to determine the effect. These results can be seen in Figure 4.27.

![Comparison of the retention of amine, carboxylate and sulfite modified beads in an aqueous suspension on porcine oesophageal tissue](image)

**Figure 4.27.** Comparison of the retention of amine, carboxylate and sulfite modified beads in an aqueous suspension on porcine oesophageal tissue.

In an aqueous suspension the amine modified beads were retained on the tissue at a higher level than the carboxylate modified beads. The retention of the amine and carboxylate modified beads appeared to be related due to the similarity in their adhesion profiles. The sulfite beads were removed from the tissue at a linear rate and the retention profile did not appear to relate to the other beads. Statistical analysis showed significant differences ($p < 0.05$) in the retention of the beads according to charge at 3, 15 and 30 minutes. Amine beads were retained to the greatest extent within an aqueous suspension, indicating that the tissue surface may possess an inherent negative charge that attracts positively charged beads to a higher degree. This negative charge may be related to the presence of mucin on the oesophageal tissue surface. Figure 4.28 compares the retention of the beads supported by the two different media at set time points.

Figure 4.28 shows the difference in the retention of the beads when supported either by alginate or water. Statistical analysis proved that in all cases the difference between the supporting media was significant with $p$ being less than 0.05 in all cases. Within the alginate solution the beads were removed from the tissue surface to a
greater extent. This result suggests that when the beads are suspended in water they are freer to interact with the tissue surface and may become more permanently attached compared to suspension within an alginate solution. This is an unexpected result as the presence of the bioadhesive carrier aimed to increase the concentration of the model drug delivered to the tissue over the designated time period. One reason for a greater interaction with tissue may be the tissue roughness. Within an aqueous suspension the beads can sink and interact with the tissue surface. The alginate, as a viscous solution, would resist this sinking motion and the beads would be removed in conjunction with the alginate solution.

![Graph showing retention of modified beads](image)

**Figure 4.27.** Comparison of the retention of modified beads suspended in alginate (solid colour) and water (striped colour)

Figure 4.29 compares 2.0 μm diameter plain and amine modified beads with covalently labelled and fluorescein-alginate solutions as a labelling technique to assess the retention of a 2 % w/v alginate solution.
Figure 4.29. Comparison of four labelling techniques

Figure 4.29 illustrates the differences noted between the retention profiles using covalently labelled alginate, solutions of alginate dispersed in sodium fluorescein plain fluorescent and amine modified beads suspended in an alginate solution as markers. The fluorescein-alginate solution and the plain fluorescent beads show very similar profiles throughout the assessment. The covalently labelled alginate is retained to a greater extent at 3 minutes although from then onwards the retention profile was similar to that of alginate-fluorescein and the plain fluorescent beads dispersed within an alginate solution. The amine modified beads were retained to a greater extent throughout the study. This result suggests that alginate labelled with fluorescein, plain fluorescent beads or a covalent attachment could be used interchangeably to assess the retention of an alginate dose. A solution of amine modified beads dispersed throughout an alginate solution may not be an appropriate system for the measurement of the retention of alginate on porcine œsophageal tissue.

4.6 CONCLUSIONS

The retention model was used to assess the extent and duration of alginate adhesion to œsophageal tissue. This method has shown results confirming the presence of a
distinct adhered layer resident on the œsophageal tissue surface. Model particles were incorporated into this adhesive layer and their retention was assessed.

The importance of the unstirred layer in the retention of an alginate dose was determined. In the absence of the unstirred water layer the alginate dose was removed from the tissue within 15 minutes. The mucin content of the washing media was not linked to the retention of the alginate dose on œsophageal tissue, thus it may not be the mucin component within the unstirred water layer holding the alginate in contact with the substrate. One theory suggests that the substrate roughness may aid the retention of the alginate on the tissue surface.

The retention of a range of alginates was investigated and the retention profiles showed a relationship between the viscosity of the alginate and the retention of the dose in the initial stages although at 30 minutes no linear trend was noted. No linear relationship was observed linking the monomeric make up of the alginate and the retention of the alginate dose at either 3 or 30 minutes.

The alginate layer was shown to be capable of supporting particles of diameters up to 2 µm. However, the surface charge of the particle was demonstrated to greatly affect the retention of the particle. Charged particles may adhere preferentially to œsophageal tissue in the absence of a bioadhesive supporting medium.
CHAPTER 5: INVESTIGATION INTO THE ADHESIVE INTERFACE USING MICROSCOPY

5.1 INTRODUCTION

As discussed in Chapter 1, many different tools have been used to investigate the adhesive interface between a bioadhesive formulation and a biological substrate. Microscopy offers the potential of visualising such an interface. However, this technique has been much overlooked in the literature, notable by the very few studies that investigate the adhesive interface using microscopy.

Lehr et al (1992) used microscopy to investigate the theories behind the bioadhesive phenomenon. Their hypothesis suggested that the interpenetration theory of bioadhesion may be visualised in a mixed system of polymer and mucin. Upon mixing a hazy or vanishing interface between the two polymeric entities would be indicative of interpenetration of the polymer chains. The results found in this study did not show such an interface, thus the conclusions drawn were that interpenetration at the mucoadhesive interface may exist although it must be restricted to the nanometer scale.

Bioadhesive formulations have been examined as individual entities using microscopy. Vitoria et al (1999) used light microscopy to investigate the effect of lecithin on poloxamer gels, used as a bioadhesive delivery system. Degim & Kellaway (1998) used light microscopy to investigate the swelling of microspheres. Swelling of such bioadhesive formulations may play an important role in the adhesive phenomenon of this type of system. Kakoulides et al (1998) used light microscopy, atomic force microscopy (AFM) and scanning electron microscopy (SEM) to investigate bioadhesive polymeric beads. These techniques were applied to beads of different formulations in order to determine their morphology. The surface properties of bioadhesive beads systems have been investigated as their porosity may greatly affect drug release from such a delivery device. Nicholls et al (1996) used light microscopy to locate the presence of lectins on corneal and
conjunctival surfaces. Their study used standard histological staining techniques that enabled the presence of the lectins to be observed and quantified.

5.2 PRINCIPLES OF MICROSCOPY

Histologists use microscopic techniques regularly as a means of assessing the depth or thickness of biological materials. However, such techniques have not been employed as a means of assessing the thickness of a bioadhesive layer. This study used light, fluorescence, confocal and atomic force microscopy as a means of measuring the depth characteristics of an alginate layer present on the œsophageal tissue surface. The principles behind these techniques are discussed in further detail within this chapter.

5.2.1 Light microscopy

Microscopy is used to provide magnification of a specimen. A simple, compound microscope enlarges the observation of a given sample. A compound microscope generates an image via two lenses. One lens, termed the objective, has a short focal length and is placed close to the object being examined. It is used to form a real image in the front focal plane of the second lens, termed an eyepiece. The eyepiece forms an enlarged virtual image that can be viewed by the observer. The magnifying power of the compound microscope is the product of the magnification of the objective lens and that of the eyepiece. A compound microscope requires an illumination system to enhance the image observed. The illumination system of the standard optical microscope is designed to transmit light through a translucent object for viewing. In a modern microscope, it consists of a light source, usually an electric lamp and a lens or lens system known as a condenser. The condenser concentrates the light, providing bright, uniform illumination in the region of the object under observation. To produce the optimum image, it is necessary to adjust the condenser such that the entire sample is brightly and evenly illuminated. The aperture of the objective lens can be adjusted to collect this light as required. Figure 5.1 shows a schematic diagram of the light microscope used in this study.
Bradbury & Bracegirdle (1998) have reviewed the principles and techniques of light microscopy in detail.

5.2.2 Fluorescence microscopy

A light microscope was used in fluorescence mode in order to visualise the fluorescently labelled alginate layer adhered to the oesophageal tissue surface. The
principle of fluorescence was explained in Chapter 4 in reference to the fluorescence spectrometer. A fluorescence microscope works in a similar way and fluorescent material is observed when light of a set wavelength hits the sample and the fluorescent molecules are excited and emit fluorescent light. Within this type of microscope a mercury lamp and filter are generally used as a means of supplying the light of a specific wavelength to illuminate the sample. One other component is required: a chromatic beam splitter or partial mirror that reflects light of lower wavelengths and allows higher wavelengths to pass. The beam splitter is important as it separates the emitted light from the excitation light. The wavelength at which the beam splitter is set allows the higher wavelengths to pass and must therefore be set according to the emission and excitation wavelengths of the fluorescent molecules used. A typical set-up is shown in Figure 5.2.

![Diagram](image)

**Figure 5.2.** Schematic representation of the principles of fluorescence microscopy

Filter sets and chromatic beam splitters are manufactured to correspond to the excitation and emission characteristics of a given fluorochrome. A book by Tanke &
Herman (1998) provides an excellent review on the principles and uses of fluorescence microscopy.

### 5.2.3 Scanning microscopes

Scanning microscopy involves the production of an image by movement of the image-forming unit over the sample and interrogating each unit on a point by point basis. The image-forming unit may be photons, electrons or an atomic force microscopy tip. Either the image-forming unit or the sample may be moved so that the points investigated are within the region of interest. Examples of scanning microscopy techniques include laser scanning confocal microscopy, scanning electron microscopy and atomic force microscopy. Scanning optical microscopy permits the acquisition of an image while only a small portion of the field is illuminated at a time. The field of view is scanned in a raster pattern, and the varying electrical signal from the photodetector is collected and stored. Since the only light passing through the sample at each point is the light used in forming each picture element, considerably higher contrast in the image is obtained. The advantages of this type of microscopy include elimination of extraneous light scattered by objects other than the illumination point.

### 5.2.4 Confocal microscopy

A confocal scanning microscope has the unique ability to produce images of a single plane, or optical section, of an object. If a series of scans are made with the microscope successively focused upon different depth planes within the object, a three-dimensional image can be reconstructed from this complete set of images. Confocal is defined as having the same focus. This means that the final image of the sample has the same focus as the object. When the object and image are confocal the microscope is able to filter out the unfocused light from above and below the point of focus in the object. Often when an object is focused using a light microscope the thickness of the sample prevents visualisation of a clear, well-focused image. The confocal microscope eliminates this out-of-focus information by means of a pin-hole situated in front of the image plane; this acts as a spatial filter and allows only the
focused portion of the light to be imaged. A diagram of the confocal principle is shown below.

![Diagram of the confocal principle]

**Figure 5.3.** Schematic representation of the confocal principle

Images seen through a confocal microscope are focused and the information has been fully filtered. This filtration process brings its own disadvantage in the loss of light within the image observed. Two components have been incorporated into the design of a confocal microscope to compensate for this loss of light. Lasers are used in place of mercury lamps as illumination sources due to the extremely bright light provided at very specific wavelengths. Very sensitive photomultiplier-detectors are also used to maximise the resolution of the image observed.

The signal is detected within a confocal microscope using a scanned focused laser beam across a square or rectangular field. A series of motorised scanner mirrors sequentially scans a horizontal beam across the specimen. The instrumentation used
allows sequential scanning in any plane. These images can be stored and superimposed as a means of producing two or three-dimensional images of a given specimen. This phenomenon is a significant advantage of confocal microscopy and it is this application that was used within this study. Further information and details about the confocal microscope are found in a book by Sheppard et al (1997).

5.2.5 Atomic force microscopy

Hammer & Tirrell (1996) examined biological adhesion at interfacial sites and suggested that only a limited number of techniques are available for the detection of an interaction between an individual receptor and a ligand. Atomic force microscopy (AFM) was included in those specialised techniques. Atomic force microscopy is able to image surfaces at very high magnifications. The principle behind AFM lies in probing the surface of a given specimen using a probe that reads the topography. The resolution of the image depends upon the size of the probe used within the investigation. Images are collected via a very fine probe that is mounted on a cantilever and scanned over the surface of a sample. This deflection forms the basic principle of image formation. According to the deflection of the cantilever the topography of a sample can be deduced. The vertical deflection of the cantilever is detected by reflecting a laser beam onto a photodiode. This is shown in Figure 5.4.

Figure 5.4. Schematic representation of the principle of atomic force microscopy
Several forces are involved in the deflection of a cantilever during AFM measurement. The force most commonly associated with AFM is the van der Waal's interatomic force. The effect of the van der Waal's force upon the distance between the tip and the sample is shown in Figure 5.5.

![Graph of interatomic force vs. distance](image)

**Figure 5.5.** Interatomic force vs. distance curve

The AFM can operate in both contact and non-contact mode, as labelled on Figure 5.5. Contact mode utilises the probe held less than a nanometer above the sample surface and the interatomic force between the sample and probe is repulsive. Non-contact mode operates via a distance of 1-10 nanometers between the specimen surface and the probe. Both these modes of operation have associated advantages and disadvantages. Contact mode was used throughout this study and is described in further detail here. In contact mode AFM the probe makes soft contact with the specimen. As the scanner rasters the tip across the sample the cantilever deflects to accommodate the topography of the sample.

Pulsed-force microscopy is a type of contact mode AFM. This technique involves tapping the probe into the specimen during a scan. This methodology allows additional properties including local stiffness and adhesion to be determined. The
resulting data is collected via a force-distance curve. A typical curve is shown in Figure 5.6.

![Figure 5.6. A typical pulsed force microscopy (PFM) force-distance curve](image)

At the start point the AFM tip is well above the specimen surface. Moving closer to the surface the tip snaps into contact with the specimen due to the negative attraction force shown in Figures 5.5 and 5.6. The tip is then pushed harder into the specimen and the positive force reaches a maximum. The resistance provided by the sample to this penetration can be correlated to the stiffness or strength of the specimen. The tip is then pulled away from the sample and the force signal reverses from repulsive to attractive due to the adhesion interaction between the tip and the specimen surface. An adherent specimen would provide greater resistance to the removal of the tip from the sample. Finally the tip is pulled away from the specimen surface and the subsequent free oscillation is drawn back to the original baseline.

This system may be able to differentiate between the physical properties of tissue and an adhesive layer. It may also provide information on the stiffness of the alginate layer as it extends from the tissue surface. This adhesivity or stiffness gradient may
be measurable using this technique. Further information about atomic force microscopy can be found in an excellent handbook by Howland & Benatar (1997).

5.3 STUDY AIMS

Microscopic techniques offer the possibility of visualisation of the alginate layer adhered to oesophageal tissue. This adhesive layer may have a role in the protection of the oesophagus as well as in the development of a novel drug delivery system targeted at this organ. This study investigates the role of light, fluorescence, confocal and atomic force microscopy in the characterisation of alginate adhesion onto porcine oesophageal tissue. Although both light and fluorescence microscopy techniques may be used to directly measure the depth of an adhered alginate layer no such techniques have been developed for confocal and atomic force microscopy. However, preliminary studies were performed using confocal and AFM in order to assess the potential of visualisation and characterisation of an adhered alginate layer on oesophageal tissue. The microscopic techniques were compared according to their ability to characterise alginate adhesion to porcine oesophageal tissue. Microscopic analysis allowed the depth of the alginate layer to be measured. The depth of the alginate layer may be related to the adhesion profile of the alginate dose on oesophageal tissue. The greater the depth of the alginate layer at set time points, the greater the retention of the alginate dose. Thus the techniques here plus those found in Chapter 4 allow the extent and duration of alginate adhesion to be quantified. The results found in both this chapter and Chapter 4 will be discussed and compared in Chapter 7, the general discussion of this thesis. Other individual aims of the different techniques will be highlighted under the relevant subheading within the method section highlighted below.

5.4 METHODS

Image tool 1.27 supplied by UTHSCSA (as described in Chapter 2) was the image analysis package used to measure the depth of the alginate layer using the different microscopic techniques. A graticule slide photographed under the same conditions as the tissue-alginate sections was used to calibrate the image analysis software. A line of known length was traced from the graticule image and set as the calibration
profile. Lines then drawn on the sectioned images could be measured according to this spatial calibration. The lines drawn on the sectioned images were taken from the uppermost edge of the alginate layer to the boundary with the tissue surface. At least 15 measurements were taken for each tissue section.

Statview version 5.0 (supplied by SAS Inc.) was used to perform statistical analyses on the results found. Analysis of variance (ANOVA) tests were performed at a significance level of 5%. Results were said to be significantly different when the p-value was less than 0.05. The details of the analyses performed were described in detail within Chapter 2.

Tissue sections from the retention model were prepared as described in Chapter 2. Sections were taken at 3 and 30 minute time points. The retention of a 2 % w/v solution of the full alginate range, LF120, SF120, LF10L, LFR5/60, SF200 and H120L was examined using all the microscopic techniques. The properties of these alginates were listed in Table 2.1 within Chapter 2. Concentration studies were also performed using; 2, 3 and 5 % w/v solutions of LF120. The effect of concentration on the depth of the alginate layer was investigated. The effect of the presence/absence of the unstirred water layer was also examined using both light and fluorescence microscopy.

The preparation of slides for light, fluorescence and AFM was described in Chapter 2. The tissue was frozen and a Shandon E Cryotome was used to take sections of approximately 15 μm. The tissue used for the confocal microscope was not sectioned but taken directly from the retention model and placed onto the confocal microscope viewing platform.

5.4.1 Light microscopy

Periodic acid-Schiff (PAS) and Alcian blue stains were applied to the tissue sections, PAS stain highlights the presence of glycoprotein (mucin) components within the tissue section, hence mucinous areas were stained purple. Alcian blue reacts with polyanions including alginate to produce a bright blue stain. The method developed by Jordan et al (1998) was used to stain the tissue sections. This technique was
described in further detail within Chapter 3. This procedure was selected as it has previously been shown to preserve the mucus layer on gastro-intestinal sections (Jordan et al, 1998). The alginate layer adherent on œsophageal tissue is a water-soluble entity and is very fragile thus a gentle technique was required. It was thought that the technique chosen might be delicate enough to preserve the alginate layer and allow imaging. It was anticipated that, as well as identifying the alginate layer to allow depth measurements, mucin presence on the tissue surface might also be visualised. Thus this technique would thus show the interface between alginate and mucin. An Olympus BX50 light microscope was used to view the tissue samples. A typical area from each specimen was selected and photographed. Image analysis was performed to assess the depth of the alginate layer in each specimen.

5.4.2 Fluorescence microscopy

Alginate solutions associated with sodium fluorescein ($\lambda_x = 490; \lambda_m = 515$ nm) were used to detect the alginate moiety adhered to the œsophageal tissue. The preparation and retention of these systems was discussed previously in Chapters 2 and 4 respectively. A Leitz Diaplan microscope was used with an N2 filter block in order to gain images of the samples. A typical region from each section was selected and photographed for image analysis. Photographs were collected under fluorescence microscopy to visualise the alginate layer. Images of the same area were taken using the light microscopy function of the microscope for comparison. The two types of image captured are compared in the results section of this chapter. The light images show the tissue yet the alginate layer is not visible thus the exact tissue surface was clearly discernible. Comparisons between the two images allowed accurate assessment of the depth of the adhered alginate layer. At least 15 measurements were taken in order to assess the depth of the alginate layer on top of the tissue surface. These measurements were used to calculate an average depth of layer plus a standard deviation for this measurement. The depth of the alginate layer according to both the concentration of the alginate solution and the physico-chemical properties of the alginate range was assessed using this technique. The depth of the alginate layer was measured and this value was correlated to the percentage of the dose remaining associated with the œsophageal tissue. This procedure was detailed in section 5.4.5 of this chapter. This analysis allowed the results obtained here to be
compared to those found in Chapter 4. A full discussion and comparison of results is given in Chapter 7.

5.4.3 Confocal microscopy

2 μm Fluoresbrite® latex microspheres (Polysciences, USA) were associated with the alginate as a means of visualising the depth of the adhered alginate layer. These beads have previously been shown to have no effect on the retention of an alginate solution as compared to sodium fluorescein. This work was shown in section 4.5.4 of Chapter 4. These microspheres were used as they possess the required excitation and emission wavelengths for use with the confocal microscope. The microscope employed was a Leica TCS SP2 microscope in conjunction with an argon ion laser. The tissue analysed was taken directly from the retention model apparatus and placed alginate layer upwards onto the confocal viewing platform. No other tissue preparation was involved. This lack of sample manipulation is a major advantage of confocal microscopy. A random area on the tissue surface was selected for analysis. The microscope scanned an area 485.75 by 485.75 μm at successive depths of 10 μm. A large z-range was investigated to ensure that the entire alginate layer was encompassed within the measurements made. A x4 objective lens was used as this lens was capable of measurements over the large z range. In using the x4 objective the axial resolution within the z-range was somewhat reduced compared with higher objective lenses. However, this set-up was considered to be the most appropriate method for the systems evaluated. This work was performed as a means of investigating the potential of confocal microscopy in the analysis of the depth of an adhered alginate layer. The results found using this technique may not be directly comparable to other techniques but will provide an indexed rank of the depth of adhered alginate layer.

5.4.4 Atomic force microscopy

Pulsed force microscopy was applied to three random areas on the alginate-tissue interface. The topography, stiffness and adhesivity profiles were measured for each specimen. These measurements allowed characterisation of the alginate-tissue
interface. One hypothesis suggests that the alginate layer may be concentrated onto the tissue surface. Measurement of the stiffness and adhesivity over this interface may provide an evidence of this concentration gradient. The range of alginates was examined to determine how the physico-chemical properties of the alginate may affect the adhesive interface. Both forward and reversing traces were taken to limit the number of artefacts seen within a given sample. The terms forward and reversing refer to the direction of travel of the probe. Certain topographical dimensions may lead to false results due to the direction of travel. The figure below highlights this phenomenon.

![Topography](image)

*Figure 5.7.* The effects of producing both a forward and reversing trace

The schematic representation in Figure 5.7 illustrates the necessity to examine both the forward and reversing trace for a given sample. Topographical artefacts may also have an impact on the results found for the adhesivity and stiffness measurements.

An area of 100 by 100 μm was examined at the tissue alginate interface. A Topometrix Explorer microscope was used in conjunction with a WITec pulsed force module. The tip used was a Topometrix SFM probe model 1620, made from FIS silicon with a frequency range of 23-38 KHz.

This technique was not used in the assessment of the depth of the adhered alginate layer. It was thought that characteristics of the adhered layer may be visualised, thus
this study was performed as a preliminary investigation into the potential of AFM to characterise the properties of an adhered alginate layer.

5.5 RESULTS AND DISCUSSION

The results were collected as images from the microscopes listed above. The different microscopic techniques were evaluated as a means of assessing the depth characteristics of the alginate layer present on the oesophageal tissue surface.

5.5.1 Light Microscopy

PAS/Alcian blue stained sections of gastric and oesophageal tissue were visualised using light microscopy and the results were shown in Figure 3.4 in Chapter 3. This work demonstrated the difference in the inherent defence systems of oesophageal tissue as compared to gastric tissue. The same techniques were applied to tissue samples that were believed to possess an adhesive alginate layer. Figure 5.8 shows sample results from this study.

![Figure 5.8](image)

*Figure 5.8.* Light microscopic images of 2 % LF120 at (a) 3 and (b) 30 minutes

In Figure 5.8 the tissue is represented by the clear, focused pink area and the alginate moiety is represented by the pale blue, wispy material. These images showed that the alginate layer was very dilute and no comprehensive layer depth measurement could be made from such an image. A more substantial alginate moiety was
observed at the 3-minute time point although the technique did not provide results of sufficient quality to justify depth measurements. The staining process had probably diluted and washed away the alginate layer to a large extent. Alginate was expected to produce a very bright blue stain with Alcian blue at a concentration of 2% w/v. This pale blue, wispy staining observed provides evidence that the slide preparation technique was not mild enough to preserve the adhered alginate layer. This technique did not provide any useful information on the characterisation of an adhesive alginate layer on oesophageal tissue and as such, no further work was performed using this technique. Although this staining procedure has previously been shown to be delicate enough to preserve the mucus layer resident on gastric and oesophageal tissue, adhered alginate did not withstand such manipulation. This result suggests that the alginate layer may therefore be less adherent to the tissue surface than comparable mucus layers.

5.5.2 Fluorescence microscopy

Tissue sections of fluorescently labelled alginate adherent on oesophageal tissue were imaged using a fluorescence microscope. Two images of each sample were taken, a fluorescent and a light image. Collection of the two images allowed accurate measurement of the depth of the adhered alginate layer. The images are presented in these results. In each of the results the fluorescent images are shown on the left hand side and comparative light images are shown on the right hand side.

5.5.2.1 Aqueous disodium fluorescein retention on oesophageal tissue

The retention of an aqueous fluorescein solution on oesophageal tissue was investigated as a means of providing a blank for the fluorescence microscopy images. This system lacks a bioadhesive carrier thus the inherent adhesivity of the fluorescent dye was measured. This system acted as a control for further investigations examining the adhesion of alginate-dye solutions on oesophageal tissue. Figure 5.9 shows the retention of aqueous sodium fluorescein on porcine oesophageal tissue. This image was taken from a sample that had been dosed for 3 minutes with 1 ml of the aqueous solution. No fluorescent layer was defined above the tissue surface thus there was no valid depth measurement as there was no adhered layer. The light
microscopic image clearly showed the tissue surface-adhered layer boundary. It was interesting to note that fluorescein had seeped into the tissue section and that fluorescence was observed throughout the section.

\textit{Figure 5.9.} Retention of an aqueous solution of sodium fluorescein on œsophageal tissue. The scale bars represent 100 µm.

Comparing the light image on the right hand side to the fluorescent image on the left hand side provides accurate visualisation of the tissue surface. If no light image was available confusion may occur between tissue surface staining and an adhered layer. No further samples were investigated for this control system as at 3 minutes, the initial time point, there was no layer visible on top of the tissue surface.

\textit{5.5.2.2 2 \% LF120 at 3 and 30 minutes}

The images shown in Figure 5.10 show the adhesion of a 2 \% w/v LF120 solution on porcine œsophageal tissue at both 3 and 30 minutes. The depth of the adhered layer was measured at each time point. A homogenous alginate layer was observed easily and the depth was measured as being 75.98 (± 10.3) µm and 59.24 (± 22.4) µm at 3 and 30 minutes respectively. The layer, as expected, was deeper at 3 minutes compared to 30. However, this difference was not significant (p > 0.05). Again it was interesting to note that fluorescein had seeped into the tissue section.
Figure 5.10. Typical fluorescence and light microscopy images of 2 % LF120 at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 μm.

5.5.2.3 2 % SF120 at 3 and 30 minutes

Figure 5.11 shows an adhered 2 % SF120 alginate layer at 3 and 30 minutes. The scale bars represent 100 μm. The depth of the adhered layer was measured at each time point. The depth of the alginate layer in this case was shown to be 79.00 (± 15.3) μm and 61.97 (± 9.7) μm at 3 and 30 minutes respectively. The difference between the depths of these adhered layers was not significant at the two time points investigated (p > 0.05).
Figure 5.11. Typical fluorescence and light microscopy images of 2% SF120 at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 µm.

5.5.2.4 2% LF10L at 3 and 30 minutes

Figure 5.12 shows a 2% w/v LF10L solution adhered to porcine oesophageal tissue. The alginate layer depth was found to be 174.63 (±12.4) µm at 3 minutes and 38.65 (±4.6) µm at 30 minutes. The difference between these depths at the two time points was found to be significant (p < 0.05).
Figure 5.12. Typical fluorescence and light microscopy images of 2 % LF10L at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 μm.

The alginate layer in this case was much thicker than expected at 3 minutes. This excessive depth may be the result of dilution and spreading of the alginate layer. This phenomenon may have occurred during sample preparation. At 30 minutes the alginate layer appeared to have two distinct parts. The layer adjacent to the tissue surface was used in the quantification of the alginate depth as this layer was continuous and immediately adjacent to the tissue surface. The second part was not considered to be part of the “real” adhered alginate and was believed to be an artefact of sample preparation, thus was ignored in assessment of the depth of the adhered alginate layer.
5.5.2.5 2 % LFR5/60 at 3 and 30 minutes

The retention of a 2 % w/v LFR5/60 solution on oesophageal tissue is shown in Figure 5.13.

![Typical fluorescence and light microscopy images of 2 % LFR5/60 at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 μm.](image)

**Figure 5.13.** Typical fluorescence and light microscopy images of 2 % LFR5/60 at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 μm.

The depth of the alginate layer was calculated to be 71.56 (± 19.4) μm at 3 minutes and 65.96 (± 15.4) μm at 30 minutes. No significant difference was noted for the retention of the dose at the different time points (p > 0.05). As expected the layer was deeper at 3 minutes compared to the corresponding layer at 30 minutes.
5.5.2.5 2 % SF200 at 3 and 30 minutes

The retention of a 2 % w/v SF200 solution is demonstrated using fluorescence microscopy. The results are shown in Figure 5.14.

![Figure 5.14](image)

*Figure 5.14.* Typical fluorescence and light microscopy images of 2 % SF200 at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 µm.

The depth of the adhered alginate layer was measured and found to be 145.77 (± 35.7) µm and 90.98 (± 18.9) µm at 3 and 30 minutes respectively. The layer is significantly thicker at the 3-minute time point as compared to the retention at 30 minutes (p < 0.05). The layer was also observed to be much more homogenous at 3 minutes compared to the layer at 30 minutes.
5.5.2.7 2% H120L at 3 and 30 minutes

The retention of 2% w/v H120L solution on oesophageal tissue was measured via fluorescence microscopy. Unfortunately no image was available for the light micrograph of the section at 30 minutes and thus the depth of the alginate layer was measured from the presumed tissue surface rather than from a more detailed guide. The observations are shown in Figure 5.15.

![Typical fluorescence and light microscopy images of 2% H120L at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 μm.](image)

**Figure 5.15.** Typical fluorescence and light microscopy images of 2% H120L at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 μm.

The alginate layer at 3 minutes appeared to have become detached from the tissue surface. The depth of this detached layer was used to calculate the depth of the layer at 3 minutes. This situation differs from the previous occurrence with 2% LF10L at
30 minutes whereby the alginate layer had split, this image shows a distinct alginate layer that was detached from the tissue surface. The alginate layer was considered to be “real” rather than an artefact. The depth of the layer was calculated to measure 171.51 (± 53.2) μm at 3 minutes. The assumed depth of the alginate layer at 30 minutes was recorded as being 151.72 (± 13.9) μm. This alginate showed extensive retention at both 3 and 30 minutes. No significant difference (p > 0.05) was noted between the alginate depths calculated at both time points.

5.5.2.8 Comparison of the alginate range

Table 5.1 compares the average depth of the adhered layer for a 2 % w/v solution of each alginate from the alginate range.

<table>
<thead>
<tr>
<th>Alginate</th>
<th>Time (minutes)</th>
<th>Count (n)</th>
<th>Mean Depth (μm)</th>
<th>Standard Deviation (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF120</td>
<td>3</td>
<td>25</td>
<td>75.98</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>37</td>
<td>59.24</td>
<td>22.4</td>
</tr>
<tr>
<td>SF120</td>
<td>3</td>
<td>19</td>
<td>79.00</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>17</td>
<td>61.97</td>
<td>9.7</td>
</tr>
<tr>
<td>LF10L</td>
<td>3</td>
<td>17</td>
<td>174.63</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>19</td>
<td>38.65</td>
<td>4.6</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>3</td>
<td>24</td>
<td>71.56</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>24</td>
<td>65.96</td>
<td>15.4</td>
</tr>
<tr>
<td>SF200</td>
<td>3</td>
<td>29</td>
<td>145.77</td>
<td>35.7</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>23</td>
<td>90.98</td>
<td>18.9</td>
</tr>
<tr>
<td>H120L</td>
<td>3</td>
<td>26</td>
<td>171.51</td>
<td>53.2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>21</td>
<td>151.72</td>
<td>13.9</td>
</tr>
</tbody>
</table>

Table 5.1. A comparison of the depth of the alginate layers at 3 and 30 minutes.

At 3 minutes LF10L, SF200 and H120L showed similar adhered depth values and no significant differences were noted between their results. However, this group of alginates showed significantly greater depths compared to the remaining three alginates, LF120, LFR5/60 and SF120. At 30 minutes H120L showed a significantly
higher depth value compared to the remaining alginites. LF10L showed a significantly lower depth value than all the other alginites except LF120. The range of alginites investigated can be grouped either by monomeric make-up with LFR5/60, SF120 and SF200 all being high G alginites or according to viscosity. LFR5/60 and LF10L are very low viscosity alginites; LF120 and SF120 are mid-range viscosity and SF200 and H120L are both high viscosity alginites. The retention of the alginites at the different time points did not appear to be affected by either of these parameters. This phenomenon was explored in further detail below. Figure 5.16 shows a comparison of the retention of the range of alginites at 3 and 30 minutes.

![Graph showing the retention of alginites at 3 and 30 minutes](image)

**Figure 5.16.** Comparison of the retention of the range of alginites as measured by fluorescence microscopy.

All the alginites demonstrated a greater alginate depth at 3 minutes as compared to 30 minutes. However, only alginites LF10L and SF200 exhibited significant differences in their retention at the two different time points investigated. (p < 0.05).

The effect of the G fraction of the alginate used was investigated in relation to the depth of the alginate layer observed. This is visualised in Figure 5.17. This figure shows that there was no relationship between the depth of the alginate layer and the G fraction of the alginate at either 3 or 30 minutes. Linear lines of best fit were applied to the data above and in both cases the regression values were less than 0.3.
This strongly suggests that no linear trend links the monomeric make up of the alginate chain to the depth/retention of the alginate.

![Graph](image)

**Figure 5.17.** Comparison of the depth of the alginate layer and the G fraction of the alginate as assessed using fluorescence microscopy

The above procedure was applied to alginates in order to determine the relationship between alginate viscosity and the depth of the alginate layer observed using fluorescence microscopy. The results are shown in Figure 5.18.

![Graph](image)

**Figure 5.18.** Relationship between alginate viscosity and the depth of the alginate layer as assessed using fluorescence microscopy
Figure 5.18 suggests a trend of increasing depth of alginate layer with an increase in the viscosity of the alginate moiety at both 3 and 30 minutes. Linear lines of best fit were applied to this data and regression values calculated. The regression values were 0.29 and 0.52 at 3 and 30 minutes respectively. No linear trend can be observed to link alginate viscosity with the depth of the adhered alginate layer.

The concentration effects of applied alginate solutions were also investigated. The depth of the alginate layer at both 3 and 30 minutes was calculated for 2, 3 and 5 % w/v solutions of LF120. The depth of the layer of 2 % LF120 was calculated as being 75.98 (± 10.3) μm and 59.24 (± 22.4) μm at 3 and 30 minutes respectively. These images were shown in Figure 5.10.

5.5.2.9 3 % LF120 at 3 and 30 minutes

The retention of a 3 % w/v solution of LF120 was measured at both 3 and 30 minutes using fluorescence microscopy. The results are shown in Figure 5.19. The alginate layer was calculated to measure 259.30 (± 45.6) μm at 3 minutes and 75.98 (± 10.3) μm at 30 minutes. A significant difference was noted between the depths of the alginate layer at the two time points (p < 0.05). The layer at 3 minutes was thicker than expected from previous results although in this case there were no signs of spreading or dilution.
Figure 5.19. Typical fluorescence and light microscopy images of 3 % LF120 at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 μm.

5.5.2.10 5 % LF120 at 3 and 30 minutes

The retention of a 5 % w/v solution of LF120 was measured at both 3 and 30 minutes. The light image of 5% LF120 at 30 minutes was underexposed and unsuitable for presentation. The fluorescence image, however is shown below and the depth measurements calculated from an estimation of the tissue surface. The results are shown in Figure 5.20.
Figure 5.20. Typical fluorescence and light microscopy images of 5 % LF120 at 3 minutes (upper images) and 30 minutes (lower images). Scale bar represents 100 μm.

The alginate layer was calculated to measure 188.65 (± 31.8) μm at 3 minutes. The assumed depth of the alginate layer at 30 minutes was recorded as being 172.15 (± 24.1) μm. This layer was very patchy and the tissue surface was considered to be very rough. No significant difference was observed between the depths of the alginate layers at the two time points (p > 0.05).
5.5.2.11 Concentration effects of alginate LF120

The depth of the alginate layers at 3 and 30 minutes are compared in Figure 5.21 for LF120 solutions at 2, 3 and 5 % w/v.

![Graph showing concentration effects of alginate LF120](image)

**Figure 5.21.** The effect of concentration on the retention of an alginate dose

Significant differences were observed between the three solutions of different alginate concentration at 3 minutes ($p < 0.05$). At 30 minutes the depth of the 5 % LF120 layer was significantly different to both 2 and 3 % LF120 solutions. Only the 3 % LF120 solution showed a significant difference in depth at the two time points investigated.

At the 30-minute time point the retention of the alginate dose appears to be dependant upon the concentration of the dose as the depth of the adhered layer increases with increasing concentration. However, at 3 minutes the retention appears to be independent of the concentration. The viscosity of the alginate solution may be related to this phenomenon. The more concentrated solutions of alginate exhibit a higher viscosity value. However, results shown in Figure 5.18 comparing the viscosity of the alginate range to the depth of the adhered layer did not exhibit any linear trends.
5.5.2.12 The effect of the unstirred water layer

The effect of the presence of the unstirred water layer was also assessed using fluorescence microscopy. Images were collected at both 3 and 30 minutes to assess the retention of a 2 % w/v solution of LF120 on natural tissue and scraped tissue where the unstirred water layer had been removed. The images from the scraped tissue can be seen in Figure 5.22.

*Figure 5.22.* Typical fluorescence and light microscopy images of the retention of 2 % LF120 on scraped tissue at 3 (upper images) and 30 minutes (lower images). Scale bar represents 100 µm.
The depth of the alginate layer was calculated to be 85.99 (± 15.0) and 74.69 (± 8.1) μm at 3 and 30 minutes respectively. No significant difference was noted between the depth measurements at the two time points investigated. This result may be compared to the result found for a 2 % w/v LF120 solution on natural tissue (images shown in Figure 5.10). The depth of the layer at 3 minutes was 75.98 (± 10.3) μm for the natural tissue and 85.99 (± 15.0) μm for the scraped tissue. At 30 minutes the depth measurements were 59.24 (± 22.4) μm and 74.69 (± 8.1) μm for the natural and scraped tissue respectively. The alginate layer on scraped tissue was measured as being deeper than the layer on normal tissue at both time points although this difference was not found to be significant (p > 0.05).

Fluorescence microscopy has provided images of the bioadhesive interface and the depth of the alginate layer was visualised and measured easily. This technique was compared to the other forms of microscopy used within this study and the relationship between the alginate used and the retention of the alginate is discussed in further detail at the end of this chapter.

5.5.3 Confocal microscopy

Confocal microscopy as a technique allowed the depth of the alginate layer to be visualised in three dimensions. Preliminary studies were performed to assess the depth of the alginate layer using this tool. The range of alginates at 2 % w/v was investigated at both 3 and 30 minutes using confocal microscopy. The depth of the alginate layer was calculated in each situation and the results compared. Images are shown for each of the alginates investigated. The depth of the alginate layer was assessed using image analysis as described previously. Fluoresbrite® beads were used to label the alginate for use with confocal microscopy.

5.5.3.1 Aqueous suspension of fluorescent beads

Confocal microscopy was performed on a blank, aqueous suspension of the fluorescent beads that were used as a fluorescent label. Images were collected at both 3 and 30 minutes and an assessment of the depth of the layer was made. This
system was a control for further measurements whereby the beads were suspended in a bioadhesive (alginate) entity. The images taken are shown in Figure 5.23.

**Figure 5.23.** Confocal microscopy shows the retention of aqueous suspension of the fluorescent beads at (a) 3 and (b) 30 minutes

The images above show the retention of an aqueous suspension of fluorescent beads as assessed using confocal microscopy. The upper images show the layer as a projection view. Within this view, the top left image represents a plan view of an
orthogonal projection of the area investigated. The legs appearing at right angles to
the image as viewed from above show the orthogonal and oblique sections of the
sample. These may be referred to as the planar images as they view the section from
both the x and y plane. Cross wires located on the square image show the area that is
investigated within the planar images. This type of representation is shown
schematically in Figure 5.24 below.

![Figure 5.24. Schematic diagram of the projection image taken from the
measurements made using the confocal microscope](image)

These images, along with the three-dimensional (3D) projection were collected for
the entire range of alginates investigated and are shown within this results section.

The lower images of Figure 5.23 show 3D representations of the layer formed by an
aqueous suspension of fluorescent beads. These images are inverted, in that the
tissue-adhered layer interface is represented by the upper surface of the labelled
layer. The 3D view provides an image of the layer as reconstructed from the many
scans taken from the successive z planes investigated. This image does not provide a
true image of the adhered layer as the beads used to mark the layer emit light both
above and below their true plane. Thus the light observed may not be contained
totally within the alginate layer. Depth measurements in this sense are comparative
with one another although the actual value may have errors associated with it. For
this reason the depth of the layer as assessed using confocal microscopy may not be correlated to the percentage of the dose retained on oesophageal tissue. Another complexity lies in the fact that the tissue surface may not be completely flat thus the image of the layer may be at an angle not represented by the data shown. This angling may lead to discrepancies in the assessment of the depth of the alginate layer. However, these are preliminary studies using confocal microscopy. The procedure may be developed and modified in future experiments to gain a more accurate visualisation of the adhered layer.

As mentioned earlier a x4 objective was used in the visualisation of the alginate layer. This lens was selected due to the extensive z-range. However, a disadvantage of this z-range was the lack of axial resolution. This means that the images were somewhat coarser than was desirable.

The control shown in Figure 5.23 shows that a fluorescent layer was present at both 3 and 30 minutes although the extent of this layer was minute in both cases. As the 3D projection did not provide a comprehensive depth view of the adhered layer examination of the orthogonal and oblique images was warranted. These images demonstrated the presence of a very small adhered layer. The 3 minute time point showed little more than a monolayer and at 30 minutes a very sparse monolayer was present. These results suggest that even at 30 minutes an aqueous suspension of beads remained associated with oesophageal tissue. Thus a bioadhesive component may not be necessary in targeting particles of this type to the oesophagus. The beads that remained associated with the tissue were present solely as a monolayer. These beads were considered to be lodged in uneven areas of the tissue surface rather than adhering due to the aqueous medium. Previous work performed using the retention model (Chapter 4, section 4.5.4) suggested that the entire dose of these beads in an aqueous suspension were removed from the tissue surface within 6 minutes.

5.5.3.2 Comparison of the retention of a range of alginates assessed using confocal microscopy

The entire range of alginates was investigated using confocal microscopy. The depth of the adhered alginate layer was calculated for all of the alginates. The images were
also compared directly and an index of alginate layer depth was compiled. Projection images for the entire alginate range are compared in Figures 5.25 and 5.26 at 3 and 30 minutes respectively.

Figure 5.25. Comparison of the projection images for the entire alginate range at 3 minutes
Figure 5.26. Comparison of the range of alginates at 30 minutes using the projection image
In some cases (e.g. SF120 in Figure 5.25) streakiness was observed in the planar images. This phenomenon is believed to be due to bead motion. The beads were suspended in a mobile liquid phase (alginate) during the image capturing process, these beads may move within this media and the motion was captured and visually represented by the streakiness observed.

Figures 5.25 and 5.26 allow comparisons to be drawn between the relative bead density associated with each adhered layer present. The depth of the alginate layer may be correlated to the density and dispersion of the beads observed in the orthogonal and oblique planar images. In themselves projection images do not allow quantitative depth measurements to be taken although depth indices can be suggested from observation.

Figure 5.25 compares the projection images at 3 minutes. The bead densities were greatest in solutions SF200 and H120L and weakest in alginates LF10L and LFR5/60. This observation suggests a relationship between alginate viscosity and depth of the adhered layer as the higher viscosity alginates displayed greater bead density. From the images displayed in Figure 5.25 the alginate range can be ranked according to their perceived depth. The order suggested was SF200 > H120L > SF120 > LF120 > LF10L > LFR5/60. This assessment is purely qualitative and has no quantitative significance.

Figure 5.26 shows the projected images of the beads suspended within the alginate range at 30 minutes. Again the bead densities were greater in the highest viscosity alginates, SF200 and H120L. The bead density did not seem to differ greatly in the remaining alginates. An index of the depth of layer was suggested from observations made from Figure 5.26; SF200 > H120L > SF120 > LF10L > LF120 > LFR5/60. The rank orders at 3 and 30 minutes suggest the same top three alginates although, as stated previously, this data is purely qualitative.

Figures 5.27 and 5.28 show the data represented in 3D at 3 and 30 minutes respectively. These images allowed quantification of the depth of the adhered alginate layer.
Figure 5.27. Three-dimensional images of alginate layers at 3 minutes
Figure 5.28. Three-dimensional confocal representation of the alginate layers at 30 minutes.
Figure 5.27 compared the depth of the adhered alginate layer from the range of alginates investigated. All the solutions were prepared at a concentration of 2 % w/v. LF120 did not show a comprehensive layer at either time point and consequently no depth measurement was made. Depth measurements were taken from the remainder of the alginate entities and these are compared in Figure 5.29.

![Graph showing depth comparison](image)

**Figure 5.29.** Comparison of the depth of the alginate layer as assessed using confocal microscopy

This figure compares the depth of layer as measured using confocal microscopy of the range of alginates at both 3 and 30 minutes. In each case the adhered layer was greater at 3 minutes compared to 30 minutes although this difference was only significant with alginate SF120 (p < 0.05). Significant differences were observed at 30 minutes between the depths measured from the two high viscosity alginates compared to the remaining alginates. The depth measurements taken here are comparative with one another yet due to the insensitive measuring technique they are not absolute values and should not be compared with the depth measurements determined using fluorescence microscopy. However, the fact that the depths noted at 3 minutes were greater than those at 30 minutes suggests that this technique has potential in the assessment of the depth of an adhered alginate layer. The actual depth values measured are listed in Table 5.2. Table 5.3 compares the qualitative...
index of alginate layer depths as assessed from the projection view to the quantitative assessment of the alginate depth determined from the 3D view.

**Table 5.2.** Comparison of the depth of the adhered alginate layer with respect to the alginate investigated.

<table>
<thead>
<tr>
<th>Alginate</th>
<th>3 minutes</th>
<th>30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF120</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SF120</td>
<td>192.74 (± 32.5)</td>
<td>79.00 (± 22.9)</td>
</tr>
<tr>
<td>LF10L</td>
<td>81.13 (± 18.7)</td>
<td>76.22 (± 15.8)</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>104.35 (± 13.6)</td>
<td>74.94 (± 15.9)</td>
</tr>
<tr>
<td>SF200</td>
<td>159.61 (± 34.4)</td>
<td>134.40 (± 15.0)</td>
</tr>
<tr>
<td>H120L</td>
<td>192.87 (± 31.4)</td>
<td>144.40 (± 19.0)</td>
</tr>
</tbody>
</table>

**Table 5.3.** A comparison of the rank order of adhered layer depth assessed using confocal microscopy

<table>
<thead>
<tr>
<th>Rank</th>
<th>3 minutes</th>
<th>30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Projection image</td>
<td>3D image</td>
</tr>
<tr>
<td>1st</td>
<td>SF200</td>
<td>H120L</td>
</tr>
<tr>
<td>2nd</td>
<td>H120L</td>
<td>SF120</td>
</tr>
<tr>
<td>3rd</td>
<td>SF120</td>
<td>SF200</td>
</tr>
<tr>
<td>4th</td>
<td>LF10L</td>
<td>LFR5/60</td>
</tr>
<tr>
<td>5th</td>
<td>LF120</td>
<td>LF10L</td>
</tr>
<tr>
<td>6th</td>
<td>LFR5/60</td>
<td>LFR5/60</td>
</tr>
</tbody>
</table>

At both 3 and 30 minutes the qualitative indices assessed from the projection images and the quantitative indices assessed using the 3D images do not correlate well. There are similarities observed in the upper and lower portions of the table although this data suggests that no estimation of alginate depth could be drawn from the qualitative data.
Further discussion about the depth of the alginate layer according to the physico-chemical properties is demonstrated below. Figure 5.36 relates the G fraction of the alginate to the depth of the alginate layer observed at both 3 and 30 minutes.

![Graph showing depth of alginate layer vs. G fraction of alginate](image)

**Figure 5.30.** Relationship between the G fraction of the alginate and the depth of the alginate layer measured using confocal microscopy

The graph above shows that there is no trend linking the monomeric make up of the alginate to the depth of the adhered alginate layer observed. In each case regression values were determined that analyse the linear line of best fit of this data, this mathematically characterises the linear relationship between the chemical composition and depth observed. At both 3 and 30 minutes the regression values were shown to be 0.35 and 0.14 respectively. These low values indicate that no linear trend was observed between the alginate composition and the depth/retention of the applied alginate dose. The viscosity of the alginate and the depth of the alginate layer were compared in a similar manner. The results are shown in Figure 5.31. The data suggested again that there was no linear trend linking the viscosity of the alginate component to the retention of the alginate dose, as assessed by depth measurement using confocal microscopy. The regression values were calculated at both 3 and 30 minutes; the values were found to be 0.29 and 0.87 respectively. This result suggests that the viscosity has a larger effect at the longer time point, although this is a very weak trend as $R^2$ is less than 0.9.
Figure 5.31. Comparison of the viscosity of the alginate and the depth of the layer measured using confocal microscopy

5.5.3.3 Concentration effects on the retention of alginate assessed using confocal microscopy

The retention of 2, 3 and 5 % w/v solutions of alginate LF120 on oesophageal tissue were assessed using confocal microscopy. Both the projection and 3D views were compared at 3 minutes in Figures 5.32 and 5.33 respectively.
Figure 5.32. Comparison of 2, 3 and 5 % alginate at 3 minutes using confocal microscopy
Figure 5.33. Comparison of the effect of alginate concentration on the 3D image of the alginate layer at 3 minutes
At 3 minutes an extensive alginate layer was observed with the 5 % w/v alginate solution in the projection view. Very low bead density was observed with the 3 % w/v alginate solution thus the rank order assessed qualitatively from these images was 5 % LF120 > 2 % LF120 > 3 % LF120. This order obviously suggests that there is no relationship linking alginate concentration with the depth of the adhered layer. Only the 5 % w/v alginate solution displayed a viable 3D image in Figure 5.33. A depth measurement was made and the depth of the alginate layer was found to be 161.58 ± 21.7 µm.

Projection and 3D images are shown for the three solutions of different concentration in Figures 5.34 and 5.35 respectively.

Figure 5.34 showed that at 30 minutes the bead density was very low for all three alginates with little more than a monolayer exhibited. These alginates were not ranked as there was no observable difference in the images.

Figure 5.35 did not show any viable 3D images from which depth measurements could be taken. No conclusions can be drawn from these results linking the depth of an alginate layer to the effect of alginate concentration as assessed using confocal microscopy.
Figure 5.34. Comparison of 2, 3 and 5 % alginate at 30 minutes using confocal microscopy
Figure 5.35. Comparison of the 3D alginate layers at 30 minutes with respect to the concentration of the alginate solution
These preliminary investigations assessed the potential of confocal microscopy as a technique to examine the depth of an adhered alginate layer on oesophageal tissue. The results found demonstrated that this technique may provide an index of results that rank alginate adhesion within a given series. Further work and modification to the system used here has great potential in providing absolute values of the depth of an adhered alginate layer. Once such a system has been developed it will provide a great deal of information on the adhesion of a liquid formulation to a biological substrate. The ability of this instrumentation to assess the depth of an adhered layer with minimal sample preparation is a major advantage of such a technique.

In summary confocal microscopy has provided a certain amount of useful information although fluorescence microscopy provided a fuller profile of the retention of an alginate dose on oesophageal tissue. The two techniques plus the others used within this chapter will be discussed more fully at the end of this chapter.

5.1.1 Atomic force microscopy

Preliminary studies were undertaken using AFM to characterise the adhered alginate layer. This technique was used to investigate the interface between the tissue surface and adhered alginate. The topography, local stiffness and adhesivity were measured on each sample selected. As stated previously this technique aimed to characterise the alginate layer and not measure the depth of the adhered alginate layer. Figure 5.36 shows the result of the AFM probing the adhesive interface.
Figure 5.36. AFM images of the interface between oesophageal tissue and alginate; (a) forward topography; (b) reverse topography; (c) stiffness and (d) adhesivity

Figure 5.36 showed parameters associated with the sample selected. The topography shows the surface profile, or shape, of the section. The tissue surface can be seen in the left hand corner of the image. This tissue is of a much higher level than the alginate layer. The height difference of the two moieties is in the range of micrometers. This height difference verges on the limit of the z-range of the instrument, thus this sample was very difficult to measure reproducibly. The alginate appeared to have a homogenous surface over the entire adhered layer. Both forward and reversing traces were taken to ensure that the images seen were “real” and did not contain topographical artefacts. In this instance the forward and reverse topographical images were similar and the image was said to be true. The stiffness and adhesivity measurements provided an almost uniform reading over the entire surface of the alginate area of the sample. However, differences were observed between the stiffness and adhesivity values of the alginate sample compared to the tissue. All of the above images were taken from the same scan of a single piece of
tissue under investigation. Figure 5.37 shows a second interface that was examined using AFM.

Figure 5.37. AFM image of a tissue-alginate interface; (a) topography; (b) stiffness; (c) forward adhesivity and (d) reverse adhesivity

The tissue surface is labelled at the right hand side of the image. Again, the topography showed a similar result to Figure 5.36 as did the stiffness and adhesivity profiles. The adhesivity is a measure of the force required to remove the probe from the sample. One hypothesis, as mentioned previously, suggested that the alginate layer may be more concentrated at the interface with the tissue surface due to specific interactions. This theory may lead to a greater stiffness noted in the alginate layer at this interfacial region. However, this effect was not observed as the adhesivity and stiffness of the alginate moiety was homogenous throughout the alginate layer. The drop-off noted at the front of the section, as presented, was an artefact of the large height difference within the sample. The tissue surface is higher.
than the alginate layer thus the image seen is "levelled". A further AFM image was taken; this is shown in Figure 5.38.

![Figure 5.38](image_url)

**Figure 5.38.** Image of the tissue-alginate interface taken using AFM; (a) topography; (b) stiffness; (c) forward adhesivity and (d) reverse adhesivity

The tissue surface was observed on the far left hand side of this image. Yet again the results were similar to those seen in Figures 5.36 and 5.37. The three AFM images shown highlight the fact that the topography and the stiffness and adhesivity of an alginate-tissue interface can be collected for a given sample. However there were many disadvantages and difficulties associated with this technique. There was a large difference in the height of the alginate as compared to the tissue. This height difference meant that the AFM instrument was working at its limits in deriving the topography as well as the stiffness and adhesivity values for the given sample. The relative stiffness and adhesivity were compared within any given sample range however, there was little difference seen across the adhesive interface. If a sample with a smaller height difference was presented to the AFM measurements may be
made on both the tissue surface and the alginate surface and comparative analyses could be drawn. The tissue stiffness or adhesivity could be normalised for all samples and the relative stiffness/adhesivity of the alginate moiety could be compared to this value. Such a set of experiments would allow the relative stiffness/adhesivity of the alginate used to be measured. These values could be used to correlate the physico-chemical properties of the alginates to the properties of the adhesive interface. In this series of experiments performed the instrument had to work at its limits to provide the images shown. As no comparative work was feasible no further experiments were performed using this apparatus. The suggested hypothesis, that the alginate layer was concentrated at the tissue surface cannot be determined from these results and further work is warranted.

The information derived from the AFM study was of limited use and the potential of this instrument in the characterisation of the alginate layer may also be limited. Extensive sample preparation is required and the freeze thaw cycle of the specimen may greatly affect the properties of the interfacial area. A great deal of further work is required in the development of this technique to enable reliable examination of the adhesive interface. However, once such a technique is available the results found would be very valuable in the characterisation of an adhered layer.

5.5.4 Validation of fluorescence microscopic techniques used to analyse the percentage of the dose adhered to the tissue

The results found in this chapter will be discussed and compared to those found in Chapter 4 in the general discussion at the end of this thesis. The results collected using fluorescence microscopy thus far have provided an index of adhesion rather than absolute values. In order to relate the depth of the adhered alginate layer to the retention model the adhered layer must be related to the percentage of the original dose applied to the tissue. A procedure was outlined for this conversion and is presented below.
1 ml (1 ml = 1000 mm³) of the dose was applied to an area of 12 by 60 mm, thus the 100% dose was considered to have a depth of:

\[ \frac{1000}{(12 \times 60)} = 1.39 \text{ mm} \]

Equation 5.1

This depth is equal to 1389 μm. To calculate the percentage depth of the original depth, which is the equivalent of the percentage of the original dose applied, the depth measured was multiplied by 100/1389 thus;

\[ \text{measured depth} \times \left( \frac{100}{1389} \right) = \% \text{ of original dose} \]

Equation 5.2

This procedure was applied to all the depth measurements calculated using both fluorescence and confocal microscopy.

A major assumption in this method relies on the fact that the applied dose measures the theoretical depth calculated using this procedure. Experiments were performed to validate this procedure.

5.5.4.1 Experimental procedure

The depth of the applied dose was not measured experimentally using microscopy as this dose flowed freely over the tissue surface. A freely flowing entity is very difficult to fix to enable reproducible microscopic analysis. These experimental procedures used a smaller, known dose dispensed onto the tissue surface to enable reproducible calculation of the depth of the adhered alginate layer. A theoretical depth value was calculated and this value was compared to the depth value calculated using the experimental techniques. Any difference in the results may be incorporated into the calculation linking the depth of the observed alginate layer to the percentage of the dose retained on the tissue surface.

0.5 ml of a 2% w/v solution of LF120 was dispensed onto a tissue section of 12 x 60 mm. This value is half of the original dose as applied in previous experiments within
this chapter. The theoretical depth of this alginate layer is equal to 694.4 μm, calculated according to equation 5.1 above, substituting 1000 mm$^3$ with 500mm$^3$, the volume of 0.5 ml. This tissue was prepared according to the methods described previously in this chapter and the depth of the layer was assessed using fluorescence microscopy.

5.5.4.2 Results

The image taken using fluorescence microscopy is shown in Figure 5.39. The depth of the alginate layer was calculated to measure 612.15 ± 84.62 μm.

![Figure 5.39. Experimental validation of the depth of the alginate layer assessed using fluorescence microscopy. Scale bar represents 200 μm.](image)

The depth of the layer as noted was smaller than the theoretical value although this difference was not significant (p > 0.05). A smaller layer was likely as the freezing involved in the sample preparation was expected to shrink the specimen and thus lead to a reduced adhered layer depth measurement. This result suggests that the images observed using fluorescence microscopy may be directly correlated to the
percentage of the alginate dose adhered to the tissue according to the calculation procedure outlined.

5.5.4.3 Confocal results

The same procedure was applied to tissue measured using confocal microscopy to note how the index of depth values relates to absolute measurements. As stated previously, confocal microscopy was investigated purely as a preliminary study and this study was used to assess the potential of this technique as a system to measure the depth of an adhered layer. figure 5.40 shows the experimental characterisation of a 0.5 ml dose applied over an area of 12 by 60 mm.

![Confocal microscopy image](image_url)

**Figure 5.40.** Experimental visualisation of the alginate layer using confocal microscopy

The depth of this layer was calculated to be 100.41 ± 16.31 μm. This value was significantly different to the theoretical value of 694.4 μm. This large difference in the experimental and theoretical values suggests that a great deal of further work is
required in refining this technique to enable absolute measurements to be made directly. A high magnification objective lens coupled with a large z-range would improve the resolution of the image obtained without limiting the range examined. Unfortunately this system was not available for this series of experiments although this factor is one means by which the results may be greatly improved. This preliminary investigation showed that confocal microscopy has potential in the characterisation of the depth of an adhered alginate layer although the results so far are only useful as comparative data within the series investigated.

The results from these validation experiments highlight the fact that confocal measurements provide an index of layer depth rather than an absolute value. In this respect fluorescence microscopy provides a more realistic profile of alginate adhesion.

In conclusion, the results found using fluorescence microscopy may be directly compared to those found using the in-house retention model. Confocal microscopy may be used in comparisons of the rank order of adhesion using a comparative index to the relative values determined using both fluorescence microscopy and the in-house retention model. Yet no relationship may be drawn between the layer depth assessed using confocal microscopy and the percentage of the original dose retained.

5.6 CONCLUSIONS

Light, fluorescence, confocal and atomic force microscopy were used as techniques to probe the adhesive interface between alginate and porcine oesophageal tissue. Light microscopy and AFM did not provide the expected results and in this instance the information provided was of little use in probing the adhesive interaction between alginate and oesophageal tissue.

The results found with both fluorescence and confocal microscopy allowed the adhered alginate depth to be measured and the alginate range to be ranked according to their relative depths. Confocal microscopy had the advantage in that the samples were not manipulated prior to measurement of the alginate depth. However, the resolution of the images obtained from confocal microscopy did not provide the
“true” picture of the alginate depth that was seen using fluorescence microscopy. Table 5.4 compares the rank order of alginate adhesion using confocal and fluorescence microscopy.

Table 5.4. Comparison of the rank order of alginate layer depth assessed using fluorescence and confocal microscopy

<table>
<thead>
<tr>
<th>Rank</th>
<th>Fluorescence microscopy</th>
<th>Confocal microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 minutes</td>
<td>30 minutes</td>
</tr>
<tr>
<td>1st</td>
<td>LF10L*</td>
<td>H120L</td>
</tr>
<tr>
<td>2nd</td>
<td>H120L</td>
<td>SF200</td>
</tr>
<tr>
<td>3rd</td>
<td>SF200</td>
<td>LFR5/60</td>
</tr>
<tr>
<td>4th</td>
<td>SF120</td>
<td>SF120</td>
</tr>
<tr>
<td>5th</td>
<td>LF120</td>
<td>LF120</td>
</tr>
<tr>
<td>6th</td>
<td>LFR5/60</td>
<td>LF10L</td>
</tr>
</tbody>
</table>

Similarities were noted in the order of alginate adhesion at both 3 and 30 minutes. The LF10L* result was unexpected as suggested in section 5.5.2.4. This layer was believed to be diffuse due to dilution and a greater depth than expected was observed. If this result is ignored comparisons may be drawn suggesting that H120L showed the largest layer depth using both techniques at both time points. SF200 is also consistently in the upper half of the table. However, there are no other discernable trends in the rank orders as assessed using the two techniques.

The absence of the unstirred layer on oesophageal tissue had no effect on the retention of the dose as assessed using fluorescence microscopy. This result contradicts the result found in Chapter 4. The general discussion in Chapter 7 compares these results.

The effect of alginate concentration on the depth of the alginate layer was not observed using either fluorescence or confocal microscopy. This result agrees with the result found in Chapter 4.
CHAPTER 6: RHEOLOGICAL INVESTIGATION

6.1 INTRODUCTION

As stated in Chapter 1 there are many methods cited in the literature that have been employed as a means of assessing mucoadhesion. Several authors have previously suggested that rheological synergism between mucin-polymer or mucus-polymer mixtures can be used as an *in vitro* parameter to determine the mucoadhesive properties of such polymers (Mortazavi et al, 1992; Rossi et al, 1995; Caramella et al, 1994). In order to discuss rheological techniques as a means of assessing adhesion it is important first of all to explain the principles behind rheology as a measurement system.

6.2 THE THEORY OF RHEOLOGY

There are two ideals found in rheology; the perfect, Hookean solid that will have 100% recovery to any applied force and also a Newtonian liquid that will flow under force yet possess no recovery. Many materials exhibit behaviour in between these two ideal situations. In order to visualise the situation, imagine a force acting on a perfectly elastic solid, the solid may compress under the action of the force but once the force is removed it will spring back to its original shape, this relates to a Hookean solid. On the other hand a perfect liquid will begin to flow in response to an applied force; once the force is removed the liquid will continue to flow indefinitely (in a frictionless system). Many pharmaceutical formulations exhibit behaviour that is between these two extremes. Creams, gels, pastes and suspensions are all examples of semi-solid materials. Rheology allows the extent of the solid and liquid like properties inherent to such materials to be quantified.

Rheology is the study of the deformation and flow of matter. The technique is used to measure the physical behaviour of a given material. Rheological measurements allow predictions to be made about a material that may be relevant to processing
and/or packaging. In basic terms rheology measures the response of a material to applied force. This is demonstrated in Figure 6.1.

![Figure 6.1](image)

**Figure 6.1.** Comparison in the behaviour of a material when it is subjected to (a) a perpendicular force and (b) a shear force

Figure 6.1 shows the application of a force to two materials. In each situation the response is also shown with compression resulting from a perpendicular force and a slide-type response from a parallel force. Rheology can measure the extent of this movement in response to an applied force and it can also measure the behaviour of the material once the force has been removed, this can be termed the recovery of a material.

There are three techniques used within rheological assessment, flow, oscillation and creep. Flow profiles assess how the motion of a liquid is affected by the strength of the force applied. These tests are useful, for example, to examine the processability of a material. Viscosity is the term used to quantify the motion of a liquid in response to a given force, a high viscosity indicates high resistance to motion whereas low viscosity materials are thin and move easily under force. In technical terms, a high viscosity material will exhibit a lower shear rate in response to a given shear stress compared to a low viscosity material. A perfect Newtonian liquid will
exhibit a constant viscosity that is independent of the magnitude of the applied force. Oscillatory tests are employed to investigate the stability of a material; these tests are useful to probe the strength of a material. Such tests measure the response of a material to a continuously applied sinusoidal stress. These oscillatory measurements allow the elastic or storage modulus, $G'$ and the viscous or loss modulus, $G''$ of a material to be assigned. Creep tests measure the response of a material to a stress; a single force is applied for a set time period and the response of the material both during the applied stress and once it has been removed are assessed. Rheological measurements are taken by applying a shear force – this is indicated in Figure 6.1 (b). In order to quantify the rheological properties of a material mathematical equations have been derived. Rheometers are capable of measuring both the force applied, and the motion of the material under test. The basic equations have been derived from these two factors.

6.2.1 Basic rheological equations

The shear stress ($\sigma$) is proportional to the force, $F$ applied (in Newtons) and inversely proportional to the area, $A$ (in m$^2$) over which the force is applied.

Shear stress, $\sigma = F/A$ (N/m$^2$ or Pascals)

Equation 6.1

Shear rate ($\gamma$) refers to the motion of the sample under the applied stress; mathematically it can be expressed velocity of the applied force (in ms$^{-1}$) divided by the thickness of the sample (in m).

Shear rate, $\gamma = v/d$ (s$^{-1}$)

Equation 6.2

The relationship between shear stress and shear rate is termed viscosity. An ideal Newtonian fluid mentioned earlier has a constant viscosity no matter what the force applied is, thus for a Newtonian liquid the viscosity ($\eta$) is defined below.
Viscosity, $\eta = \frac{\text{shear stress}}{\text{shear rate}} = \frac{\sigma}{\gamma}$ (N/m².s⁻¹ or Pa.s)  

Equation 6.3

These equations are very basic but it is from these principles that the more complex models and behaviour predictions arise. The experimental work within this chapter used the flow function of the rheometer, thus, flow experiments will be described in greater detail here. This project is concerned with the retention of a liquid system within the oesophagus. Liquid systems are usually measured in the flow profile as they move in response to stress and have little recovery. Both oscillatory and creep methods are better suited to materials that have a greater solid-type component as they are both interested in the recovery of a material after an applied stress. In this instance the formulation is required to flow within the oesophagus yet also be retained within this organ, for this reason flow is the most appropriate technique.

Flow is usually performed in order to determine the viscosity of a material. The results are shown as flow curves. These curves show shear stress against shear rate, typical examples are shown in Figure 6.2. Alternatively the data can be shown as viscosity against shear rate.

Figure 6.2. Typical flow curves
This type of graph can be thought of as plotting the strength of the force applied, \( \sigma \) against the motion of the sample, \( \gamma \). Materials can be grouped according to their rheological behaviour under stress. These representative groups are shown in the example flow curve above. The Newtonian liquid gives a straight line passing through the origin; this represents the fact that the viscosity is unchanged by shear stress. The viscosity of a material is given by the gradient of a shear stress versus shear rate flow curve. Both Bingham and Plastic materials are required to overcome an initial resistance to flow prior to any motion. This is shown on the graph where there is no corresponding rate value for initial shear stress values. Both the plastic and the pseudoplastastic material show non-constant gradients with a decline. This decline in gradient occurs at increased values of shear rate and shear stress. The behaviour is alternatively described as shear thinning, in that the viscosity is reduced with high forces or high rates of shear. This performance is commonplace; consider paint, a material that is thick until stirred vigorously. Dilatant materials show the opposite effect with the viscosity increasing with shear. An example of a dilatant material is double cream that thickens when stirred. As many materials have viscosity values that change according to shear, it is important to compare their viscosity values under identical conditions. A set shear rate or stress value is used to draw comparisons. Alternatively the viscosity values over a range are compared. It is also important to use the same temperature when making rheological assessments as the viscosity of many materials alters with temperature.

Shear rate values can be stated to correspond to particular events. Table 6.1, taken from Barnes et al (1989); lists the different rates involved in diverse processes. When considering the application of an adhesive system targeted at the œsophagus the shear rates involved correspond to those for chewing and swallowing and also draining under gravity. A shear rate range of 0.1 – 100 s\(^{-1}\) was thus investigated.
Table 6.1. Shear rates of some familiar materials and processes.

<table>
<thead>
<tr>
<th>Situation</th>
<th>Typical shear rate range ($s^{-1}$)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation of fine powders in a suspending liquid</td>
<td>$10^{-6}$-$10^{-4}$</td>
<td>Medicines, paints</td>
</tr>
<tr>
<td>Levelling due to surface tension</td>
<td>$10^{-2}$-$10^{-1}$</td>
<td>Paints, printing inks</td>
</tr>
<tr>
<td>Draining under gravity</td>
<td>$10^{1}$-$10^{1}$</td>
<td>Painting and coating</td>
</tr>
<tr>
<td>Extruders</td>
<td>$10^{0}$-$10^{2}$</td>
<td>Polymers</td>
</tr>
<tr>
<td>Chewing and swallowing</td>
<td>$10^{1}$-$10^{2}$</td>
<td>Foods</td>
</tr>
<tr>
<td>Dip coating</td>
<td>$10^{1}$-$10^{2}$</td>
<td>Paints, confectionary</td>
</tr>
<tr>
<td>Mixing and stirring</td>
<td>$10^{1}$-$10^{3}$</td>
<td>Manufacturing liquids</td>
</tr>
<tr>
<td>Pipe flow</td>
<td>$10^{0}$-$10^{3}$</td>
<td>Blood flow</td>
</tr>
<tr>
<td>Spraying and brushing</td>
<td>$10^{3}$-$10^{4}$</td>
<td>Spray drying, painting</td>
</tr>
<tr>
<td>Rubbing</td>
<td>$10^{4}$-$10^{5}$</td>
<td>Application of creams etc to the skin</td>
</tr>
<tr>
<td>Lubrication</td>
<td>$10^{3}$-$10^{7}$</td>
<td>Engines</td>
</tr>
</tbody>
</table>

6.2.2 The rheological apparatus

The rheometer used within this project was a TA Instruments (UK) AR 1000N controlled stress rheometer. A schematic diagram representing this instrument is shown in Figure 6.3. The basic mechanism of action of the rheometer is that the draw rod is rotated or oscillated at a set shear rate or stress according to the method chosen. This draw rod is rotated via a motor. In order to reduce the friction this motor operates via an air bearing so that the force applied can be measured as accurately as possible. In running an experiment the sample is placed on the Peltier plate taking care to minimise the disruption caused to this sample. The geometry is then lowered slowly so that it is in contact with the sample. The draw rod then drives the geometry controlling either the speed or the force with which geometry is rotated. The response to this motion is recorded as sample data.
Several geometries are available according to the sample and procedure chosen. Two typical examples are (a) parallel plates, whereby the gap can be set to an arbitrary value, and (b) cone geometries. These geometries are not strictly conical as they have been truncated. The gap set between the plates and the cone must be exactly equal to the truncation value of the cone. The advantages of cones are that they have a larger surface area and are thus more suited to the measurement of lower viscosity samples. All geometries come in a variety of sizes (diameters) and again larger diameters increase the surface area and are thus more appropriate for low viscosity samples. This study used a 6 cm diameter 2° acrylic cone geometry with a truncation value of 58 μm.

**Figure 6.3.** Schematic representation of the rheometer used.
6.2.3 Rheology as a technique to assess bioadhesion

Rheology has been suggested as being a useful tool in probing the interaction between a biological substrate and an adhesive polymer as the rheological properties can be linked to the interpenetration theory of bioadhesion (Mortazavi et al 1993). In general, it is mucoadhesion rather than bioadhesion that has been extensively investigated using rheological techniques. In the majority of cases the interaction between mucin solutions or mucus and an adhesive formulation have been examined. Mucin as a chemical entity has been discussed previously in Chapters 2 and 3. Rheological tools are useful in probing mucoadhesion due to the assumption that interpenetration occurs between polymers and mucin macromolecules and that this interpenetration is the basis of the rheological change that occurs in a mixed system. The interpenetration theory states that the mucus chains from the biological substrate become entangled with the polymeric chains of the adhesive formulation. This synergy is noted in mixed systems. Rheological methods do not provide a comprehensive explanation for the mucoadhesive interaction. However, the method provides a simple and accurate means of ranking a range of mucoadhesive polymeric moieties.

Hassan & Gallo (1990) performed a simple viscometric assay to assess the interaction of polymers and mucin as a means of predicting the bioadhesive nature of a polymeric formulation. Other studies in a similar vein have also been performed; these include studies by Mortazavi et al (1992) who looked at the storage ($G'$) and loss moduli ($G''$) of a mixed system of mucin and polyacrylic acid. It has been proposed that if the moduli are increased in the mixed system compared to those expected from the individual components an interaction has occurred, which is indicative of good mucoadhesive performance.

In order to assess rheological synergism simple equations are used:

$$\Delta \eta = \eta \text{ (mix)} - \{ \eta \text{ (mucin)} + \eta \text{ (polymer)} \}$$

Equation 6.4

where:
\[ \eta_{\text{mix}} = \text{apparent viscosity (Pa.s)} \text{ of the polymer-mucin mixture} \]
\[ \eta_{\text{mucin}} = \text{apparent viscosity (Pa.s)} \text{ of the mucin dispersion having the same concentration as in the mixed system} \]
\[ \eta_{\text{polymer}} = \text{apparent viscosity (Pa.s)} \text{ of the polymer solution having the same concentration as in the mixed system} \]

One of the disadvantages of this methodology is that the mucus or mucin used may not be fully representative of the mucosal substrate \textit{in vivo}. Various studies have compared a range of polymeric materials and ranked them in their mucoadhesive potential (Mortazavi & Smart, 1995; Caramella et al, 1994; Madsen et al, 1998). However all these studies investigated just one type of mucus. Other studies have investigated the adhesive properties of polymeric formulations in contact with different mucin-type materials. Rossi et al (1999) investigated two different commercially available types of porcine gastric mucin in combination with sodium carboxymethylcellulose. No difference in the results was noted between the mucin samples and thus the author concluded that commercial mucin in this case was satisfactory. Mortazavi et al (1993) compared crude mucus, homogenised mucus and purified mucus glycoproteins in their ability to interact with polyacrylic acid. This polymer had previously shown synergistic interactions in combination with homogenised porcine gastric mucus. The results showed no difference in the interaction with the three biological substrates according to the mucin concentration of the substrate. However, it is important to note that extraction and purification of mucin can lead to many changes in the inherent structure of this material that may lead to discrepancies within the results collected. A paper by Rossi et al (1995) highlights the diverse effects noted by the use of different mucin substrates on the mucin-polymer interaction. This difference noted, demonstrates one of the disadvantages involved in using \textit{in vitro} rheological techniques as a means of grading the mucoadhesive potential of such polymers. Rossi et al (1995) investigated the differences found between commercially prepared mucin and in house preparations the main advantages and disadvantages are listed in the following table.
Table 6.2. The advantages and disadvantages associated with the use of commercial mucin as compared to in-house prepared mucin.

<table>
<thead>
<tr>
<th>Mucin Source</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial Mucin</td>
<td>Lower batch to batch variability.</td>
<td>Preservation processes including freeze drying or freezing may alter the physical properties of the mucin samples.</td>
</tr>
<tr>
<td></td>
<td>Ready to use.</td>
<td></td>
</tr>
<tr>
<td>Freshly prepared</td>
<td>The processes applied to the mucin are known.</td>
<td>Samples are prepared from relatively few animals.</td>
</tr>
<tr>
<td>mucin</td>
<td>Mucin is not subjected to preservation processes.</td>
<td>The sample has a limited life span.</td>
</tr>
</tbody>
</table>

Rossi et al (1995) investigated the synergy between polymers including, polyacrylic acid and sodium carboxymethylcellulose, and three different types of commercially available mucin. Porcine gastric mucin (PGM) and bovine submaxillary mucin (BSM) were supplied by Sigma, UK. The third mucin was porcine gastric mucin supplied by Prodotti Gianni, Italy. The results showed no synergy for polyacrylic acid in combination with any of the three mucins investigated. Sodium carboxymethylcellulose showed a greater degree of synergy in combination with BSM as compared to both PGM samples. This study highlights the importance of mucin selection when using rheology as a tool to probe the mucoadhesive interaction. It is important to use the appropriate mucin in relation to the target site for adhesion.

Alginate has been investigated in combination with mucin and the mucoadhesive potential of this polymer has been ranked by several authors. Mortazavi & Smart (1995) ranked alginate highly in combination with crude hog mucus gel through tensile testing. However rheological testing of alginate in combination with homogenised porcine mucus gel did not show extensive synergism (Madsen et al, 1998). Fuongfuchat et al (1996) investigated alginate in combination with purified ovine and porcine submaxillary mucin. The results showed significant interaction between alginate and both ovine and porcine submaxillary mucins. These results
suggest that the synergy observed may be dependant on the nature of the experimental conditions, the chemicals used and the concentrations of these chemicals.

6.3 AIMS OF THE STUDY

The mucoadhesive potential of a range of alginates was investigated within this chapter. Previous work performed demonstrated the presence of mucin within the unstirred water layer that resides on porcine oesophageal tissue. It is unclear as to whether this mucin is derived from swallowed saliva, oesophageal secretions or refluxed gastric mucus. Rheological synergism was used as a test to determine the mucoadhesive potential of alginate in combination with porcine gastric mucin, bovine submaxillary mucin and also in combination with both artificial and natural saliva as well as the unstirred water layer as removed from oesophageal tissue. Chapter 3 described further characterisation of both mucin solutions, saliva (artificial and natural) and the oesophageal scrapings. Chapter 4 showed that the retention of an alginate dose on oesophageal tissue was dependant upon the presence of the unstirred layer. However, results in Chapter 5 showed that the alginate adhesion was not affected by the presence/absence of the unstirred water layer. Alginate is investigated with components of the unstirred layer as a means of probing the interaction between alginate and this biological substrate. Rheological synergy may provide an insight into the real value of this unstirred water layer in the adhesion of an alginate solution.

6.3.1 Materials used in the rheological study

The range of alginates used included LF120, SF120, LF10L, LFR5/60, SF200 and H120L. The properties of these alginates are listed in Table 2.1 of Chapter 2. A 2 % w/v solution of each of the alginates was prepared as well as a 3 and 5 % solution of alginate LF120. The more concentrated solutions were investigated as a means of noting the effect of alginate concentration on the synergy observed with a biological substrate. The biological substrates investigated included porcine gastric mucin (PGM), bovine submaxillary mucin (BSM), natural saliva, artificial salivas I and II
and also the unstirred water layer removed from the oesophageal tissue surface as oesophageal scrapings. 2 % solutions of both porcine gastric mucin and bovine submaxillary mucin were prepared. Natural saliva was collected according to the method described in Chapter 2, from a single, healthy female volunteer and used as required. The two artificial salivas used were prepared as described in Chapter 2. 2, 3, 5 and 10 % solutions of porcine gastric mucin were also prepared in order to investigate the effect of mucin concentration on the interaction with alginate.

The unstirred water layer was removed from oesophageal tissue in the form of oesophageal scrapings. This procedure was also described in Chapter 2.

6.3.2 Rheological procedure

Each individual component was mixed in a 1:1 ratio (or other relevant ratio) with deionised water prior to measurement in a mixed system with either alginate or mucin. This dilution was performed to ensure that the concentration of each component in the mixed system was equal to the concentration of the material when measured alone. The mixed systems were prepared by weighing the required amount of each component into a glass vial. This vial was then stirred vigorously for approximately 60 seconds with a narrow glass rod. Each sample was allowed to stand for at least 30 minutes prior to measurement. This equilibration period ensured that the forces involved in the mixing process did not affect the rheological profile of the material.

A suitable volume (approximately 2 mls) of each sample was gently placed onto the Peltier plate of the rheometer ensuring minimal disruption to the sample. Once the sample had been loaded the geometry was lowered slowly into contact with the sample at a logarithmic rate until the geometry reached the designated point. This action acted to minimise the disruption to the sample. A six-centimetre diameter, 2° acrylic cone geometry was used for all the experiments, the truncation value was 58 μm. The large diameter ensured that the measurement of even the lower viscosity solutions was as accurate as possible. The method used was a controlled shear ramped experiment. The shear rate range investigated was 0.1 – 100 s⁻¹. This range
was selected because it encompasses the shear rate values quoted for swallowing and draining under gravity (Barnes et al 1989). A set shear rate value of 10 s\(^{-1}\) was used to compare the viscosity values of the samples measured. This shear rate value was selected due to its physiological relevance; a shear rate of 10 s\(^{-1}\) is encompassed both within the range quoted for swallowing and also for draining under gravity. This type of experiment measured the equilibrium viscosity of the sample at set shear rate values within the range of 0.1-100 s\(^{-1}\). All the experiments were performed at 37 °C. Each experiment was performed a minimum of 4 times and the mean value and standard deviation was calculated.

6.4 RESULTS AND DISCUSSION

As stated in equation 6.4 rheological synergism is measured by calculating the difference between the theoretical and experimental viscosity value of the mixed system.

\[ \Delta \eta = \eta \text{(mix)} - \{ \eta \text{(mucin)} + \eta \text{(polymer)} \} \]

*Equation 6.4*

The theoretical viscosity value was calculated by adding the viscosity values of each individual component. This calculation was performed over the entire shear rate range and plotted as a profile, alternatively a particular shear rate value was chosen for the calculation. Positive synergy, which is indicative of an interaction, is present when \(\Delta \eta\) is positive.

6.4.1 Alginate range in combination with 2% PGM

The range of alginates was investigated in combination with a 2 % w/v solution of PGM. An example of the data collected is shown in Figure 6.4. The 2 % solution of LF120 is shown over the entire shear rate range in a 1:1 ratio with 2 % PGM.
Figure 6.4. Alginate in combination with porcine gastric mucin

Figure 6.4 shows the viscosity of a 1 % PGM (dark blue) and a 1 % LF120 alginate solution (pale blue) as well as the additive, theoretical viscosity (shown in red). The experimental data is also shown (black). The theoretical viscosity was greater than the experimental value at low shear rates indicating negative synergy, however, at higher shear rates the experimental viscosity was greater than the theoretical value. This figure demonstrates the form in which the rheological data was collected. This data set was collected from each mixed system investigated. The results collected at a shear rate of 10 s⁻¹ were used as comparative data within the alginate range investigated. Figure 6.5 shows a comparison of the experimental and theoretical viscosity values for the full range of alginates investigated. This data is shown at a fixed shear rate of 10 s⁻¹.
Figure 6.5. Comparison of the theoretical and experimental viscosity values for a range of alginates in combination with 2 % PGM solution

This graph shows positive synergy with some alginates and negative synergy with others. Positive synergy is demonstrated when the experimental data is greater than the theoretical data values. Table 6.3 shows the percentage change in the experimental viscosity compared to the theoretical value.

Table 6.3. A measure of the synergy found between a range of alginates and 2 % PGM solution

<table>
<thead>
<tr>
<th>Alginate used</th>
<th>Normalised theoretical viscosity (%)</th>
<th>Comparative experimental viscosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF120</td>
<td>100</td>
<td>130.32 (±11.42)</td>
</tr>
<tr>
<td>SF120</td>
<td>100</td>
<td>104.46 (± 14.30)</td>
</tr>
<tr>
<td>LF10L</td>
<td>100</td>
<td>112.75 (± 60.28)</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>100</td>
<td>82.20 (± 39.36)</td>
</tr>
<tr>
<td>SF200</td>
<td>100</td>
<td>171.27 (± 2.63)</td>
</tr>
<tr>
<td>H120</td>
<td>100</td>
<td>74.63 (± 4.55)</td>
</tr>
<tr>
<td>3%LF120</td>
<td>100</td>
<td>200.17 (± 5.93)</td>
</tr>
<tr>
<td>5%LF120</td>
<td>100</td>
<td>89.68 (± 20.66)</td>
</tr>
</tbody>
</table>
The data in Table 6.3 was manipulated so that the theoretical data was normalised to 100 %. The experimental data was then processed to give a percentage value corresponding to the theoretical data. This comparative experimental data value can also be termed the extent of synergy. A value less than 100 % indicates negative synergy whereas a value greater than 100 % shows positive synergy and an interaction between the two components investigated.

The alginates investigated can be grouped either according to monomeric make-up or according to viscosity. Figure 6.6 shows a scatter plot relating the extent of synergy to the G-fraction of the alginate.

![Scatter plot relating the extent of synergy to the G-fraction of the alginate](image)

**Figure 6.6.** Relating the G fraction of the alginate to the extent of synergy observed with a 2 % PGM solution

The relationship between the G fraction of the alginate and the extent of synergy can be observed in Figure 6.6. The results noted may suggest that the synergy was greater for alginates with a low G fraction. However, no linear trend was observed, $R^2 < 0.9$.

The alginate range can also be grouped according to viscosity, LF10L and LFR5/60 have very low comparative viscosity values; SF120 and LF120 are mid range and both SF200 and H120L are high viscosity alginates. The scatter plot shown as Figure 6.7 represents the relationship between alginate viscosity and the extent of synergy observed. The alginate viscosity plotted is the viscosity of a 2 % solution of each of the alginate measured at a shear rate of 10 s$^{-1}$ at 37 °C.
Figure 6.7. The relationship between the viscosity of the alginate and extent of synergy in mixed systems of alginate and PGM

Figure 6.7 shows the relationship between the synergy observed for a mixed system of alginate with 2% PGM and the viscosity of the alginate component. The results show no linear relationship between the extent of synergy and alginate viscosity ($R^2 < 0.9$).

The results observed when alginate and porcine gastric mucin are mixed in a 1:1 ratio show no obvious trends according to the physico-chemical properties of the alginate range investigated.

6.4.2 Alginate in combination with 2% BSM

The same procedure as in section 6.4.1 was applied to a 2% solution of bovine submaxillary mucin in place of the porcine gastric mucin solution. The results were collected in the same way. Figure 6.8 shows a comparison of the viscosity of a 1% solution of both BSM and LF120 as well as the experimental and theoretical data for the 1:1 ratio mixed system.
Figure 6.8. Comparison of the experimental and theoretical viscosity profiles for 2% LF120 in a 1:1 ratio with 2% BSM

The figure above shows each data point collected with standard deviations. The viscosity of the 1% solutions of BSM and the alginate are again plotted and the theoretical data calculated. This theoretical data can be directly compared with the data found experimentally. Figure 6.9 shows the comparative data at a set shear rate of 10 s\(^{-1}\) for the full range of alginates investigated.

Figure 6.9. A comparison of the experimental and theoretical viscosity values for the 1:1 ratio of mixed alginate-2% BSM solution
This graph shows the experimental and theoretical data for 1:1 mixed systems of alginate and bovine submaxillary mucin. Positive synergy is observed where the experimental data values are greater than the theoretical values.

Table 6.4 compares the normalised data for the experimental results as compared to the theoretical data.

**Table 6.4.** The synergy seen with the range of alginates in combination with 2% BSM

<table>
<thead>
<tr>
<th>Alginate</th>
<th>Normalised theoretical viscosity (%)</th>
<th>Comparative experimental viscosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF120</td>
<td>100</td>
<td>112.42 (±10.43)</td>
</tr>
<tr>
<td>SF120</td>
<td>100</td>
<td>159.33 (±65.35)</td>
</tr>
<tr>
<td>LF10L</td>
<td>100</td>
<td>166.95 (±95.95)</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>100</td>
<td>53.77 (±13.94)</td>
</tr>
<tr>
<td>SF200</td>
<td>100</td>
<td>109.52 (±33.99)</td>
</tr>
<tr>
<td>H120</td>
<td>100</td>
<td>79.87 (±19.03)</td>
</tr>
<tr>
<td>3% LF120</td>
<td>100</td>
<td>289.58 (±117.07)</td>
</tr>
<tr>
<td>5% LF120</td>
<td>100</td>
<td>73.88 (±15.80)</td>
</tr>
</tbody>
</table>

Scatter plots were prepared to show the trend between the extent of synergy and the physico-chemical properties of the alginate range. Figure 6.10 shows the relationship observed between the alginate G fraction and the extent of synergy in combination with 2 % BSM. The results suggest that there is no observable trend linking the alginate G fraction and the extent of synergy observed in these mixed systems.
**Figure 6.10.** Trend between alginate G fraction and the extent of synergy in a mixed system of alginate and BSM

The trend between alginate viscosity and the extent of synergy between alginate and BSM is shown in Figure 6.11.

**Figure 6.11.** Relationship between alginate viscosity and the extent of synergy observed in mixed systems of alginate and BSM

No linear relationship was observed linking the extent of synergy and the alginate viscosity in the mixed systems investigated. Mixed systems of alginate and 2 % BSM solutions showed no relationship between the extent of synergy according to the physico-chemical properties of the alginate range investigated.

### 6.4.3 Alginate in combination with natural saliva

The range of alginates was investigated in combination with natural saliva. The natural saliva used, was collected as required from a healthy female volunteer. The
mucin content of this saliva was measured as discussed in Chapter 3. The saliva was mixed in a 1:1 ratio with water and measured alone prior to measurement in a 1:1 ratio with the alginate. This ensured that the results were directly comparable. As with the previous two sections the same series of experiments were performed using natural saliva as the biological substrate. The full viscosity profile of natural saliva in combination with 2 % LF120 is shown in Figure 6.12.

![Figure 6.12. Rheological profile of natural saliva in combination with 2 % LF120](image)

This data shows that a mixed system of natural saliva and LF120 has experimental and theoretical profiles that are closely matched. Figure 6.13 compares the difference in the experimental and theoretical viscosity values found for the range of alginates in combination with natural saliva. All the measurements were made at a shear rate value of 10 s⁻¹. Positive synergy is again shown when the experimental data values are greater than the corresponding theoretical data values.
Figure 6.13. The synergy observed for a range of alginates in combination with natural saliva

Again these results show no specific trend in the synergy patterns according to the range of alginates investigated. The numerical values associated with the synergy observed within this study are highlighted in Table 6.5.

Table 6.5. A comparison of the synergistic interactions between a range of alginates and natural saliva

<table>
<thead>
<tr>
<th>Alginate</th>
<th>Normalised theoretical viscosity (%)</th>
<th>Comparative experimental viscosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF120</td>
<td>100</td>
<td>119.35 (± 7.96)</td>
</tr>
<tr>
<td>SF120</td>
<td>100</td>
<td>110.94 (± 37.56)</td>
</tr>
<tr>
<td>LF10L</td>
<td>100</td>
<td>387.44 (± 125.19)</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>100</td>
<td>276.12 (± 200.83)</td>
</tr>
<tr>
<td>SF200</td>
<td>100</td>
<td>73.73 (± 43.00)</td>
</tr>
<tr>
<td>H120</td>
<td>100</td>
<td>157.86 (± 102.72)</td>
</tr>
<tr>
<td>3%LF120</td>
<td>100</td>
<td>201.58 (± 96.90)</td>
</tr>
<tr>
<td>5%LF120</td>
<td>100</td>
<td>77.12 (± 13.55)</td>
</tr>
</tbody>
</table>
Figure 6.14 shows the relationship between the G fraction of the alginate range and the extent of synergy observed between alginate and natural saliva in a 1:1 ratio.

**Figure 6.14.** Trend observed between the extent of synergy and the alginate G fraction in mixed systems of alginate and natural saliva

The figure above shows that there is no relationship linking the extent of synergy and the alginate G fraction. A similar plot is shown in Figure 6.15. This plot compares the alginate viscosity to the extent of synergy in mixed systems of alginate and natural saliva.

**Figure 6.15.** Relationship between the extent of synergy observed and the viscosity of the alginate in mixed alginate natural saliva systems

The figure above shows a slight trend between the alginate viscosity and the extent of synergy observed. The extent of synergy appears to be reduced at high viscosity
values. However, there was no linear relationship noted, $R^2 < 0.9$. The synergism observed in mixed systems of alginate and natural saliva showed no distinct trends according to the physico-chemical properties of the alginate.

### 6.4.4 Alginate in combination with artificial saliva I

Artificial saliva I was prepared according to the formulation in Chapter 2. This was mixed in a 1:1 ratio with deionised water and measured alone. The artificial saliva as prepared was then mixed in a 1:1 ratio with each of the range of alginites and their rheological profiles were assessed. Figure 6.16 shows the rheological profiles for artificial saliva I in combination with water as well as in combination with a 2% solution of LF120.

![Figure 6.16. Rheological profile of 2% LF120 in a 1:1 ratio with artificial saliva I](image)

The theoretical data for this mixed system shows a higher viscosity value throughout the majority of the shear rate range compared to the experimental result indicating negative synergy. However, it is interesting to note the very low viscosity values of the artificial saliva in combination with water. The uneven trace observed is an artefact of the very low viscosity as the rheometer is measuring at the limits of its capacity. Figure 6.17 shows the difference in the theoretical and experimental viscosity values for the entire alginate range in a 1:1 ratio with artificial saliva I.
Figure 6.17. A comparison of the experimental and theoretical viscosity values for 1:1 mixed systems of alginate and artificial saliva I.

Extensive negative synergy was observed for mixed systems of alginate in combination with artificial saliva I. The numerical data is shown in Table 6.6.

Table 6.6. Synergistic interactions between an alginate range and artificial saliva I

<table>
<thead>
<tr>
<th>Alginate</th>
<th>Normalised theoretical viscosity (%)</th>
<th>Comparative experimental viscosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF120</td>
<td>100</td>
<td>65.35 (± 3.18)</td>
</tr>
<tr>
<td>SF120</td>
<td>100</td>
<td>78.23 (± 12.13)</td>
</tr>
<tr>
<td>LF10L</td>
<td>100</td>
<td>77.50 (± 10.55)</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>100</td>
<td>75.09 (± 7.84)</td>
</tr>
<tr>
<td>SF200</td>
<td>100</td>
<td>105.38 (± 8.21)</td>
</tr>
<tr>
<td>H120</td>
<td>100</td>
<td>36.90 (± 5.66)</td>
</tr>
<tr>
<td>3% LF120</td>
<td>100</td>
<td>140.14 (± 3.22)</td>
</tr>
<tr>
<td>5% LF120</td>
<td>100</td>
<td>78.20 (± 8.81)</td>
</tr>
</tbody>
</table>

Scatter plots are shown in Figures 6.18 and 6.19 illustrating the trends observed for the extent of synergy relating to the physico-chemical properties of the alginates.
investigated. Figure 6.18 relates the extent of synergy to the G fraction of the alginate entity.

![Graph showing the relationship between G fraction of alginate and extent of synergy.](image)

**Figure 6.18.** Relationship between the G fraction of the alginate and the extent of synergy observed in mixed systems of alginate and artificial saliva I

The figure above shows no trend between alginate G fraction and the extent of synergy observed in the mixed systems investigated. Figure 6.19 compares the viscosity of the alginate component and the extent of synergy detected.

![Graph showing the relationship between viscosity and extent of synergy.](image)

**Figure 6.19.** Relationship between the extent of synergy detected and the viscosity of the alginate moiety in mixed systems of alginate and artificial saliva I

The relationship between alginate viscosity and the extent of synergy in mixed systems of alginate and artificial saliva I was not observed from the data displayed in Figure 6.19. This result in addition to the result found above suggest that no trend is
observed between the extent of the synergy seen and the physico-chemical properties of the alginate range.

### 6.4.5 Alginate in combination with artificial saliva II

Artificial saliva II was prepared as described in Chapter 2. This was mixed in a 1:1 ratio with both water and the alginate range in order to perform a series of experiments that would allow the interaction between the two components to be measured. The experiments performed were the same as the previous section. Figure 6.20 shows the rheological profile for a 1:1 ratio of artificial saliva II and 2% LF120.

![Rheological profile of a 1:1 mixed system of artificial saliva II and 2% LF120](image)

**Figure 6.20**. Rheological profile of a 1:1 mixed system of artificial saliva II and 2% LF120

This figure illustrates negative synergy between 2% LF120 and artificial saliva II. Again the very low viscosity of the artificial saliva was difficult to measure using the instrumentation available. The range of alginates in a 1:1 ratio with this artificial saliva were examined, the results are shown in Figure 6.21.
This figure shows negative synergy in the majority of cases between the alginate and artificial saliva mixed systems. The theoretical viscosity values are greater than the experimental values in almost all instances. These results are also displayed in a normalised form in Table 6.7.

**Table 6.7.** A comparison of the synergy observed between a range of alginates and artificial saliva II

<table>
<thead>
<tr>
<th>Alginate</th>
<th>Normalised theoretical data (%)</th>
<th>Comparative experimental data (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF120</td>
<td>100</td>
<td>34.46 (± 3.42)</td>
</tr>
<tr>
<td>SF120</td>
<td>100</td>
<td>92.51 (± 7.06)</td>
</tr>
<tr>
<td>LF10L</td>
<td>100</td>
<td>143.40 (± 7.79)</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>100</td>
<td>92.08 (± 5.36)</td>
</tr>
<tr>
<td>SF200</td>
<td>100</td>
<td>70.80 (± 6.44)</td>
</tr>
<tr>
<td>H120</td>
<td>100</td>
<td>77.87 (± 6.03)</td>
</tr>
<tr>
<td>3% LF120</td>
<td>100</td>
<td>86.17 (± 24.38)</td>
</tr>
<tr>
<td>5% LF120</td>
<td>100</td>
<td>33.75 (± 10.22)</td>
</tr>
</tbody>
</table>
Scatter plots are shown in Figures 6.22 and 6.23. These plots relate the physico-chemical properties of the alginate to the extent of synergy observed in mixed systems of alginate and artificial saliva II.

Figure 6.22. Relationship between the G fraction of the alginate and the extent of synergy observed in mixed systems of alginate and artificial saliva II

No relationship was observed between the alginate G fraction and the extent of synergy according to the results shown above. The relationship between alginate viscosity and the extent of synergy is demonstrated in Figure 6.23 below.

Figure 6.23. Trend between the alginate viscosity and extent of synergy observed in mixed systems of artificial saliva II and alginate

Again no relationship was noted between reduced alginate viscosity and the extent of synergy measured in the mixed systems investigated. No significant trends were
observed between the physico-chemical properties of the alginate range investigated and the extent of synergy observed in mixed systems of alginate and artificial saliva II.

Table 6.8 collates all the data found for the range of alginates in combination with the substrates investigated. The table shows the presence (Y) or absence (N) and also the extent of synergy as well as the numerical values and standard deviations. In certain cases no significant result (~) could be observed and this is also noted on the table.

Comparing the entire range of alginates with the synergy measured did not provide any observable trends in the interactions between the alginates and the substrates tested. Porcine gastric mucin and natural saliva showed a greater number of synergistic interactions compared to bovine submaxillary mucin and both artificial saliva formulations. The percentage mucin present in the artificial saliva formulations was 0.25 and 0.27 w/v % for formulations I and II respectively. This low mucin concentration may account for the fewer observable interactions. The interaction as related to the mucin content of a sample was investigated in further detail later in this chapter. The relationships examining the extent of synergy observed according to the physico-chemical properties of the alginate showed no significant trends.
Table 6.8. An evaluation of the comparative experimental data demonstrating the extent of synergy observed between the alginate range and substrates tested

<table>
<thead>
<tr>
<th>Alginate</th>
<th>PGM</th>
<th>BSM</th>
<th>Natural saliva</th>
<th>Artificial saliva I</th>
<th>Artificial saliva II</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF120</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>130.32</td>
<td>112.42</td>
<td>119.35</td>
<td>65.35</td>
<td>34.46</td>
</tr>
<tr>
<td></td>
<td>(11.42)</td>
<td>(10.43)</td>
<td>(7.96)</td>
<td>(3.18)</td>
<td>(3.42)</td>
</tr>
<tr>
<td>SF120</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>104.46</td>
<td>159.33</td>
<td>110.94</td>
<td>78.23</td>
<td>92.51</td>
</tr>
<tr>
<td></td>
<td>(14.30)</td>
<td>(65.35)</td>
<td>(37.56)</td>
<td>(12.13)</td>
<td>(7.06)</td>
</tr>
<tr>
<td>LF10L</td>
<td>~</td>
<td>~</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>112.75</td>
<td>166.95</td>
<td>387.44</td>
<td>77.50</td>
<td>143.40</td>
</tr>
<tr>
<td></td>
<td>(60.28)</td>
<td>(95.95)</td>
<td>(125.19)</td>
<td>(10.55)</td>
<td>(7.79)</td>
</tr>
<tr>
<td>LFR5/60</td>
<td>~</td>
<td>N</td>
<td>~</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>82.20</td>
<td>53.77</td>
<td>276.12</td>
<td>75.09</td>
<td>92.08</td>
</tr>
<tr>
<td></td>
<td>(39.36)</td>
<td>(13.94)</td>
<td>(200.83)</td>
<td>(7.84)</td>
<td>(5.36)</td>
</tr>
<tr>
<td>SF200</td>
<td>Y</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>171.27</td>
<td>109.52</td>
<td>73.73</td>
<td>105.38</td>
<td>70.80</td>
</tr>
<tr>
<td></td>
<td>(2.63)</td>
<td>(33.99)</td>
<td>(43.00)</td>
<td>(8.21)</td>
<td>(6.44)</td>
</tr>
<tr>
<td>H120</td>
<td>N</td>
<td>N</td>
<td>~</td>
<td>~</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>74.63 (4.55)</td>
<td>79.87</td>
<td>157.86</td>
<td>36.90</td>
<td>77.87</td>
</tr>
<tr>
<td></td>
<td>(19.03)</td>
<td>(102.72)</td>
<td>(54.66)</td>
<td>(6.03)</td>
<td></td>
</tr>
<tr>
<td>3% LF120</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>~</td>
</tr>
<tr>
<td></td>
<td>200.17</td>
<td>289.58</td>
<td>201.58</td>
<td>140.14</td>
<td>86.17</td>
</tr>
<tr>
<td></td>
<td>(5.93)</td>
<td>(119.07)</td>
<td>(96.90)</td>
<td>(3.22)</td>
<td>(24.38)</td>
</tr>
<tr>
<td>5%LF120</td>
<td>~</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>89.68</td>
<td>73.88</td>
<td>77.12</td>
<td>78.20</td>
<td>33.75</td>
</tr>
<tr>
<td></td>
<td>(20.66)</td>
<td>(15.81)</td>
<td>(13.55)</td>
<td>(8.81)</td>
<td>(10.22)</td>
</tr>
</tbody>
</table>
6.4.6 Concentration effects of the alginate according to the synergy observed

LF120 alginate solutions of 2, 3 and 5 % w/v were investigated in 1:1 ratios with the entire substrate range noted. The data collected was shown on the previous figures alongside the data for the alginate range. The trends observed according to the alginate concentration are shown in Figure 6.24 below.

![Graph showing concentration effects of alginate](image)

**Figure 6.24.** Comparison of the extent of synergy according to the concentration of the alginate solution. (PGM = porcine gastric mucin; BSM = bovine submaxillary mucin; NS = natural saliva; AS I = artificial saliva I and AS II = artificial saliva II)

The figure above shows the effect of the concentration of the alginate on the extent of synergy observed. As the alginate concentration increases the viscosity of the alginate also increases. Previous results have shown that the extent of synergy observed with all the biological substrates used was not affected by the viscosity of the alginate component. The 3 % LF120 exhibited the largest synergistic effect with all of the biological substrates investigated. The extent of synergy observed for the 2 and 5 % solutions was similar in each case. These results suggest that an optimum alginate concentration may be necessary to produce a large synergistic effect. Only a very small range of alginate concentrations was investigated within this study, thus
conclusions drawn relate to a very small population. It would be interesting to investigate this effect over a greater range of alginate concentrations.

6.4.7 2% LF120 in combination with natural saliva at a range of ratios

Natural saliva collected from a single, healthy female volunteer was investigated at a range of ratios in combination with 2 % LF120. A full rheological profile for this mixed system in a 1:1 ratio can be seen in Figure 6.12. A comparison of the experimental and theoretical viscosity values at a set shear rate of 10 s⁻¹ are shown in Figure 6.25.

![Viscosity Comparison](image)

Figure 6.25. A comparison of the viscosity values for the experimental and theoretical data for the mixed alginate – natural saliva systems investigated

These results are also recorded in Table 6.9. The table allows a comparison of the extent of the synergy noted for the mixed systems discussed above.
Chapter Six: Rheological Investigation

**Table 6.9.** A comparison of the degree of synergy between mixed systems of alginate and natural saliva

<table>
<thead>
<tr>
<th>Mixed system</th>
<th>Normalised theoretical data (%)</th>
<th>Comparative experimental data (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:3 saliva:2% LF120</td>
<td>100</td>
<td>145.58 (± 10.58)</td>
</tr>
<tr>
<td>1:1 saliva:2% LF120</td>
<td>100</td>
<td>151.22 (± 36.82)</td>
</tr>
<tr>
<td>3:1 saliva:2% LF120</td>
<td>100</td>
<td>162.17 (± 25.22)</td>
</tr>
</tbody>
</table>

The figure and table above demonstrate that the extent of synergy increased as the percentage of saliva in the mixed system increased. This result indicates that saliva may have less binding sites available for interaction than alginate as a greater proportion of saliva is required to induce extensive synergy. Again it would be interesting to investigate a greater range of mixed systems as it is a very small population from which these results are drawn.

### 6.4.8 Concentration effects of PGM on the synergy observed with alginate

2 % LF120 was investigated in a 1:1 mixed system with porcine gastric mucin dispersions prepared at a range of different concentrations. 2, 3, 5 and 10 % solutions of PGM were prepared as described in Chapter 2. A typical trace of 2 % LF120 in a 1:1 ratio with 2 % PGM is shown in Figure 6.4. Figure 6.26 highlights the experimental and theoretical viscosity data found for the mixed systems at a set shear rate of 10 s⁻¹. This figure illustrates the relationship between alginate and PGM. As the mucin concentration increases the degree of synergy also increases. This phenomenon is also shown in Table 6.10.
Figure 6.26. A comparison of the experimental and theoretical data collected for 1:1 ratios of LF120 in combination with solutions of PGM

Table 6.10. Comparison of the synergy seen in mixed systems of PGM and alginate

<table>
<thead>
<tr>
<th>Mixed system</th>
<th>Normalised theoretical data (%)</th>
<th>Comparative experimental data (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% mucin:2% LF120</td>
<td>100</td>
<td>142.82 (± 22.11)</td>
</tr>
<tr>
<td>3% mucin:2% LF120</td>
<td>100</td>
<td>168.32 (± 13.54)</td>
</tr>
<tr>
<td>5% mucin:2% LF120</td>
<td>100</td>
<td>185.27 (± 9.23)</td>
</tr>
<tr>
<td>10% mucin:2% LF120</td>
<td>100</td>
<td>216.86 (± 11.04)</td>
</tr>
</tbody>
</table>

This table highlights the fact that the extent of synergy increased with an increase in the percentage of the mucin component. This relationship has been investigated and is highlighted in Figure 6.27.
Figure 6.27. A measure of the extent of synergy in an alginate-PGM mixed system

Figure 6.27 plots the degree of synergy measured at a shear rate $10 \text{ s}^{-1}$ between solutions of porcine gastric mucin and 2 % LF120. An equation of best fit has been added to this plot, it may be used to determine the linear relationship between mucin concentration and the extent of synergy observed. A trend is noted between increasing mucin concentration and the extent of synergy observed. This trend is strong as $R^2 > 0.9$. The equation shown on the graph describes the relationship between the extent of synergy ($y$) and the mucin concentration ($x$) of a mixed system.

6.4.9 2% LF120 in combination with œsophageal scrapings

The surface layer resident on the œsophagus was removed via a gentle action with a scalpel. This material was mixed in a 1:3, 1:1 and 3:1 ratio with 2 % LF120 and the rheological profile was assessed. Figure 6.28 shows the rheological profile for the scrapings in a 1:1 ratio with water, 1 % LF120 and also the mixed system. The theoretical data for the mixed system is also shown.
Little synergy was observed between the oesophageal scrapings and the alginate solution. This experiment was repeated at ratios of 1:3 and 3:1 to compare the rheological profiles of the scrapings in combination with the alginate. In each case the two individual components were mixed in the relevant ratios with water to ensure that the results were directly comparable. These results were taken at a set shear rate of 10 s⁻¹ and are shown in Figure 6.29.

**Figure 6.28.** The rheological profile for 2% LF120 in combination with oesophageal scrapings

**Figure 6.29.** A comparison of the synergy seen in mixed systems of porcine oesophageal scrapings and 2% LF120
Figure 6.29 shows clearly that positive synergy was seen between the low concentration scrapings and the 1:1 ratio of scrapings with alginate although from the figure, the extent of synergy can be seen to be very small in the 1:1 ratio mixed system. Negative synergy is observed for the 3:1 ratio of scrapings:alginate. Table 6.9 shows the extent of synergy seen as a percentage of the normalised theoretical data for each of the mixed systems investigated.

*Table 6.11.* A measure of the synergy detected in mixed systems of LF120 and porcine oesophageal scrapings

<table>
<thead>
<tr>
<th>Mixed system</th>
<th>Normalised theoretical viscosity (%)</th>
<th>Comparative experimental viscosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:3 scrapings:2% LF120</td>
<td>100</td>
<td>535.62 (± 7.52)</td>
</tr>
<tr>
<td>1:1 scrapings:2% LF120</td>
<td>100</td>
<td>129.48 (± 3.79)</td>
</tr>
<tr>
<td>3:1 scrapings:2% LF120</td>
<td>100</td>
<td>92.81 (± 5.62)</td>
</tr>
</tbody>
</table>

These results indicate that the extent of synergy is greater in systems where the ratio of scrapings to alginate is low. This suggests that there are fewer interaction sites present on oesophageal tissue as compared to the alginate moiety. The mixed system rapidly becomes saturated with alginate, as this saturation occurs no further interaction is feasible thus no further synergy is observed.

The mucin content of the biological substrates investigated was calculated and the relationship between mucin content and the extent of alginate synergy was investigated. Table 6.12 shows the data.
Table 6.12. Correlation of the mucin concentration of the biological substrate and the degree of synergy seen. *Measured as the average mucin content of one individual's saliva over two time points Data from Chapter 3. †According to results found in Chapter 3, the concentration of mucin within oesophageal scrapings as determined according to a PAS assay using PGM as a control.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mucin content</th>
<th>Extent of synergy</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 % PGM</td>
<td>2 %</td>
<td>130.32 (± 11.4)</td>
</tr>
<tr>
<td>2 % BSM</td>
<td>2%</td>
<td>112.42 (± 10.4)</td>
</tr>
<tr>
<td>Natural saliva</td>
<td>0.62 % *</td>
<td>119.35 (± 8.0)</td>
</tr>
<tr>
<td>Artificial saliva I</td>
<td>0.25</td>
<td>65.35 (± 3.2)</td>
</tr>
<tr>
<td>Artificial saliva II</td>
<td>0.27</td>
<td>34.46 (± 3.4)</td>
</tr>
<tr>
<td>Oesophageal scrapings</td>
<td>0.12 % †</td>
<td>129.48 (± 3.8)</td>
</tr>
</tbody>
</table>

The data shown in Table 6.12 was plotted on a scatter diagram to observe the relationship between mucin content and the extent of synergy observed in a 1:1 ratio with 2 % LF120. The results are shown in Figure 6.30.

Figure 6.30. Relationship between the extent of synergy of 2 % LF120 and the mucin content of the range of biological substrates investigated

This data shows that negative synergy was observed with 2 % w/v LF120 and the two artificial saliva formulations. The extent of synergy observed with oesophageal...
scrapings, natural saliva and both mucin solutions was similar. These results suggest that the mucin concentration of the substrate may not be fully responsible for the synergistic interactions observed and that many other factors may also be involved. This result contradicts the result found in the investigation into the effect of increasing the PGM concentration in a 1:1 ratio with 2% LF120.

6.5 CONCLUSIONS

The importance of the unstirred layer in the retention of an alginate dose was shown in Chapter 4. Results in Chapter 5 suggested that the retention of the alginate dose was not affected by the absence of the unstirred water layer. This chapter investigated the rheological synergy between alginate solutions and the biological substrates associated with adhesion within the oesophagus. Synergy was observed in mixed systems of alginate and oesophageal scrapings however the extent of this synergy was weak. Rheological techniques do not appear to relate to the adhesive phenomenon of alginate adhesion to porcine oesophageal tissue.
CHAPTER 7: GENERAL DISCUSSION

7.1 INTRODUCTION

The experimental work performed so far within the project has been discussed at the end of the relevant chapters. This chapter aims to draw together universal discussion about the nature of the work and the characteristics of the adhesive alginate layer. The results found will be concluded on a global scale and suggestions will be made for future work.

The aims of this project included characterisation of alginate adhesion onto oesophageal tissue. The rationale behind this work involved the development of an adhesive formulation that may act as a protective mechanism for delicate oesophageal tissue with a secondary application as a drug delivery system.

The study was divided into two sections; the characterisation of oesophageal tissue surface as the target site of adhesion and experimental methods developed to probe the interaction observed between alginate and oesophageal tissue. Four situations were investigated within the experimental procedures; the effect of the presence/absence of the unstirred layer resident on the surface of oesophageal tissue, the effect of the alginate physico-chemical properties on the interaction, the effect of alginate concentration on the retention observed and the ability of the alginate layer to support model drug particles. This chapter aims to discuss each of these situations and summarise the results found throughout this project.

7.1.1 Analysis of the unstirred water layer resident on oesophageal tissue

Very little work has been performed investigating the unstirred water layer as an entity, thus analysis of the composition and characteristics of this material was performed. Previous studies investigated the depth of such a layer and also the pH gradient through the layer. The depth measurements differed between studies performed with depth values of 30 and 95 \( \mu \text{m} \) being suggested by Attwood (1994)
and Sarosiek (1994) respectively. The presence of a mucus layer on oesophageal tissue has been the subject of much debate. Some studies (Tanaka et al, 1996; Namiot et al, 1994) have demonstrated the presence of mucus on oesophageal tissue whereas other studies (Nunn et al, 1990) demonstrated the presence of only bacteria on oesophageal tissue surfaces in the rat. Initial studies therefore focused on proving the existence of mucin components within the unstirred layer and quantifying the concentration of these mucus components.

The first experimental chapter, Chapter 3, investigated the nature of the surface properties of porcine oesophageal tissue. Two methods were used to quantify the depth of the unstirred water layer, followed by biochemical and histological techniques to determine and quantify the presence of mucin within the unstirred water layer.

The depth of the unstirred layer was calculated using both microscopic and mass analysis techniques. The depth was calculated to be 107.3 μm using mass analysis and 33.0 μm using microscopy. These results were found to be significantly different using analysis of variance (ANOVA) tests at a significance level of 5 %. However, previous work has suggested that the depth unstirred water layer was 30 μm (Attwood, 1994) and 95 μm (Sarosiek, 1994). The values determined within this study correlate well to those found in previous studies.

PAS/Alcian blue staining techniques exhibited the presence of mucin within the unstirred water layer. This mucin was quantified and found to be present at a concentration of less than 0.3 % w/w. This value correlates well with a previously determined value suggested by Dixon (1997).

Saliva was also considered an important part of the adhesion phenomenon between alginate and oesophageal tissue. Physiologically the oesophagus is continually washed with saliva, thus this fluid may have an important role in the adhesion of any substance to the oesophagus. The unstirred water layer resident on oesophageal tissue may be partly derived from swallowed saliva and in this respect it was interesting to also determine the mucin content of saliva. The mucin concentration within saliva...
was determined at a range from 0.5 – 2.4 % w/v. This level was greater than expected as literature values suggested the maximum concentration to be 0.6 % w/v (Lentner, 1981). However, this difference was discussed in Chapter 3. It was interesting to note that the mucin concentration in saliva and in œsophageal scrapings were of similar levels. This result adds merit to the theory that the unstirred water layer may consist of swallowed saliva.

7.1.2 Investigation into the role of the unstirred water layer in the retention of the alginate dose

The role of the unstirred water layer in the retention of the alginate dose was investigated using the retention model, microscopy and also rheological techniques. The retention model showed that the absence of the unstirred water layer led to very reduced retention of the alginate dose with the entire dose being removed from the tissue within 15 minutes compared to greater than 20 % of the original dose remaining on the tissue for 30 minutes on natural (unscraped) tissue. Results obtained using fluorescence microscopy contradicted this finding. The depth of the layers noted at both 3 and 30 minutes were similar for scraped and natural tissue.

Rheological investigations were performed to investigate the components of this unstirred layer in combination with alginate. Mucin solutions, artificial saliva formulations, natural saliva and the œsophageal scraping themselves were investigated in combination with alginate. It has been suggested previously that rheological synergy is an indication of an interaction between two components in a mixed system. Hassan & Gallo (1990) suggested that an increase in the viscosity of a mixed system of polymer and mucin as compared to the viscosity values of the individual components may be indicative of the bioadhesive potential of the polymer under investigation. Rheological synergism was assessed at a physiologically relevant shear rate and the results found are outlined below. Synergy was observed between solutions of alginate and solutions of mucin. This synergy appeared to increase as the concentration of mucin increased. Negative synergy was observed with solutions of artificial saliva, although their mucin content was very low. Both natural saliva and œsophageal scrapings (the unstirred layer removed from the tissue surface) showed synergy in combination with alginate. The mucin content of the
biological substrate under assessment did not appear to affect the extent of synergy observed indicating that other material present in the unstirred layer may be accountable for the interaction observed between alginate and oesophageal tissue.

This study suggested that the unstirred water layer and alginate interact to a certain extent although the retention of the alginate dose may not be entirely dependant on the mucin content within this unstirred water layer.

7.1.3 Investigation into the effect of the alginate physico-chemical properties on the retention of an alginate dose on oesophageal tissue

A range of alginates was investigated whose properties were listed in Table 2.1 within Chapter 2. The retention of these alginates was determined using both fluorescence microscopy and in-house retention techniques. The fluorescence microscopy results are directly comparable to the in-house model as suggested by the validation experiments performed in section 5.5.4 of Chapter 5. The relationship between the retention of the alginate and the monomeric make-up as well as the viscosity of the alginate solutions was investigated. Figure 7.1 compares the retention of the alginate dose as assessed using the in-house model and fluorescence microscopy at the 3 minute interval.

![Figure 7.1. Comparison of the retention of an alginate dose at 3 minutes as assessed using the in-house model and fluorescence microscopy](image-url)
The data in Figure 7.1 shows the percentage of the original dose remaining on the oesophageal tissue as assessed by each technique. The results showed that the actual amount of the dose retained on the tissue was much larger when the assessment was made using the in-house retention model, compared to the microscopic technique. The in-house retention model allowed measurements to be taken under physiologically relevant conditions of greater than 90 % RH and at 37 °C. Fluorescence microscopy measurements were performed using frozen, sectioned tissue, thus some degree of shrinkage was expected.

It is important to note that the in-house retention model measures the percentage of the dose washed off the tissue whereas fluorescence microscopy measured the dose retained on the tissue. Thus, when comparing the results certain assumptions have been made. It was assumed using the in-house retention model that no labelled material was lost in the analytical procedure. Thus, the entire washings were collected and anything not collected remained associated with the tissue surface. Fluorescence microscopy showed that fluorescence was observed within the tissue specimen on top of which an alginate layer was adhered. This suggests that some of the fluorescent material applied to the surface seeped into the tissue and thus reduced the fluorescence in the collected fraction. This implies that the amount of the dose remaining on the tissue surface may be an overestimation of the true value. The validation experiment performed for fluorescence microscopy in Chapter 5 suggested that although there was no significant difference in the experimental value of the measured layer, 612.15 (± 84.6) µm compared to the theoretical value of 694.4 µm the mean experimental value measured was somewhat reduced. From these results it may be suggested that fluorescence microscopy underestimated the layer depth by approximately ten percent. However, even accounting for these considerations the results still show differences in the retention of an alginate dose as assessed using fluorescence microscopy compared to the in-house retention model.

Table 7.1 compares the rank order of alginate adhesion according to the in-house retention model, fluorescence microscopy and confocal microscopy at 3 minutes. The depth values assessed using confocal microscopy were found to be valid as a
relative index although correlation to the percentage of the original dose applied was not deemed to provide meaningful results.

Table 7.1. Comparison of the rank order of alginate adhesion using three different techniques at 3 minutes

<table>
<thead>
<tr>
<th>Rank</th>
<th>In-house model</th>
<th>Fluorescence microscopy</th>
<th>Confocal Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>SF200</td>
<td>H120L</td>
<td>H120L</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>H120L</td>
<td>LF10L</td>
<td>SF120</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>LF120</td>
<td>SF200</td>
<td>SF200</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>SF120</td>
<td>SF120</td>
<td>LFR5/60</td>
</tr>
<tr>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
<td>LF10L</td>
<td>LFR5/60</td>
<td>LF10L</td>
</tr>
<tr>
<td>6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>LFR5/60</td>
<td>LF120</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.1 compares the three techniques used to rank the depth of the adhered alginate layer. At the 3 minutes time point there was little similarity in the rank orders for each of the techniques. No correlation was determined using any of the techniques to link the linear relationship between the G fraction of the alginate range and the extent of adhesion. The in-house retention model did show a linear trend between alginate viscosity and the extent of adhesion at 3 minutes ($R^2 > 0.9$). However, neither microscopic technique exhibited any relationship between alginate viscosity and the depth of the adhered layer.

The retention of the alginate dose at 30 minutes was also investigated using the in-house retention model and fluorescence microscopy and the results are compared in Figure 7.2.
Figure 7.2. Comparison of the retention of the alginate dose as assessed using the in-house retention model and fluorescence microscopy at 30 minutes

As with the results at 3 minutes the in-house model generally gave much larger values for the retention of the alginate dose. However, the alginate doses retained to the least extent, LF10L and LFR5/60, showed comparable results between the two techniques. The differences in retention between the two techniques appear to be smaller at the 30 minutes time point as compared to the results shown in Figure 7.1 at 3 minutes. The errors associated with the in-house retention model are much greater than those determined using fluorescence microscopy due to the different analysis techniques. The large errors associated with the in-house retention model were discussed fully in Chapter 4.

Again, the ranked order of the retention of the alginates was compared at 30 minutes. The 30 minute time point was considered to be less dependant upon the experimental technique and thus the results collected may be compared with greater confidence. In this instance rheological techniques were also compared to the in-house retention model, fluorescence and confocal microscopy. Rheological results ranked the according to their extent of synergy observed in combination with 2 % w/v porcine gastric mucin. The other techniques ranked the alginates according to their observed retention. These results are shown in Table 7.2.
Table 7.2. Comparison of the rank order of alginate adhesion using four different techniques at 30 minutes

<table>
<thead>
<tr>
<th>Rank</th>
<th>In-house model</th>
<th>Fluorescence microscopy</th>
<th>Confocal Microscopy</th>
<th>Rheology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>H120L</td>
<td>H120L</td>
<td>H120L</td>
<td>SF200</td>
</tr>
<tr>
<td>2nd</td>
<td>SF120</td>
<td>SF200</td>
<td>SF200</td>
<td>LF120</td>
</tr>
<tr>
<td>3rd</td>
<td>SF200</td>
<td>LFR5/60</td>
<td>SF120</td>
<td>LF10L</td>
</tr>
<tr>
<td>4th</td>
<td>LF120</td>
<td>SF120</td>
<td>LF10L</td>
<td>SF120</td>
</tr>
<tr>
<td>5th</td>
<td>LFR5/60</td>
<td>LF120</td>
<td>LFR5/60</td>
<td>LFR5/60</td>
</tr>
<tr>
<td>6th</td>
<td>LF10L</td>
<td>LF10L</td>
<td></td>
<td>H120L</td>
</tr>
</tbody>
</table>

Table 7.2 shows that the three techniques that assess alginate retention provide very similar results at 30 minutes with H120L being retained to the greatest extent in all three situations. These three techniques also show similarities at the bottom of the table with LF10L and LFR5/60 generally being retained the least. Rheological results do not show the same degree of similarity, the largest difference being noted with H120L being bottom of the table compared to top in all the other cases.

These results suggest that the four techniques investigated show very different profiles of alginate adhesion to oesophageal tissue. Few similarities were observed between the techniques noted. This result may be an artefact of each technique assessing a different aspect of the alginate oesophageal tissue interaction. Alternatively these results may be due to the fact that there is no correlation linking the alginate properties to the adhesion of the alginate dose. At 30 minutes no linear trends were noted linking the alginate G fraction or viscosity to the extent of adhesion using any of the techniques investigated. From these results there is no definitive rank order expected for alginate adhesion and the difference in rank orders noted is an artefact of this phenomenon.

It is interesting to note that all three techniques that assessed alginate retention displayed an adhesive alginate layer for at least 30 minutes. Rheological studies
showed positive synergy for mixed systems of alginate in combination with low concentrations of the unstirred water layer as removed from the tissue surface.

Further rheological assessments were made investigating the range of alginates in combination with many biological substrates. These biological substrates were selected due to their relevance in the adhesion of a system on oesophageal tissue. Each substrate appeared to interact with the alginate range in a different way and the ranked orders of alginate interaction were dissimilar throughout the range of substrates investigated. None of the biological substrates investigated showed any linear trend linking either the monomeric structure of the alginate or its viscosity to the extent of synergy observed.

The techniques discussed above were shown to be capable of assessing the extent and duration of an adhesive system on oesophageal tissue. These techniques did not correlate as well as was hoped although they all produced results that were clear and repeatable.

7.1.4 Investigation into the effect of the alginate concentration on the retention of an alginate dose on oesophageal tissue

The retention of three alginate solutions; 2, 3 and 5 % w/v LF120 were investigated using the in-house retention model and fluorescence microscopy. Figure 7.3 shows how the retention of the alginate was affected by both the concentration and the technique used to assess the retention at 3 minutes.
Figure 7.3. The effect of concentration on the retention of an alginate as assessed using the in-house retention model and fluorescence microscopy at 3 minutes.

The figure above shows that the retention of the alginate dose as assessed using the in-house retention model shows a strong trend linking the retention of the dose and the concentration of the given dose. Fluorescence microscopy did not show a significant trend linking the concentration of the alginate dose and the retention of the alginate at 3 minutes. Figure 7.4 shows the retention of the alginate doses at 30 minutes.

Figure 7.4. The effect of concentration on the retention of an alginate as assessed using the in-house retention model and fluorescence microscopy at 30 minutes.
Chapter Seven: General Discussion

The retention of the doses as assessed using the in-house retention model suggested that no trend linked the concentration of the alginate dose to the retention of the dose. Fluorescence microscopy suggested that as the concentration of the alginate dose increased the extent of retention also increased. Table 7.3 compares the rank order of alginate adhesion according to the concentration of the alginate solution as assessed using fluorescence microscopy and the in-house retention model and the extent of synergy noted between the alginate solutions and a 2\% solution of porcine gastric mucin.

Table 7.3. Comparison of the rank order of alginate adhesion according to concentration using three different techniques

<table>
<thead>
<tr>
<th>Rank</th>
<th>In-house retention model</th>
<th>Fluorescence microscopy</th>
<th>Rheology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 minutes</td>
<td>30 minutes</td>
<td>3 minutes</td>
</tr>
<tr>
<td>1st</td>
<td>5 % LF120</td>
<td>2 % LF120</td>
<td>3 % LF120</td>
</tr>
<tr>
<td>2nd</td>
<td>3 % LF120</td>
<td>5 % LF120</td>
<td>5 % LF120</td>
</tr>
<tr>
<td>3rd</td>
<td>2 % LF120</td>
<td>3 % LF120</td>
<td>2 % LF120</td>
</tr>
</tbody>
</table>

This table shows that the rank order of alginate adhesion is again dependant on the technique used to assess this adhesion.

Further rheological assessments were again performed investigating the effects of concentration of the alginate moiety with the extent of synergy observed in combination with a variety of biological substrates. In each case the 3 \% alginate solution appeared to interact to the greatest extent. This result suggested that an optimum concentration of alginate may be required to interact with the biological substrates investigated.
7.1.5 Investigation into the effect of the alginate concentration on the retention of an alginate dose on œsophageal tissue

Model drug particles of a variety of size and charge were investigated in this study. The ability of the alginate layer to support such particles was investigated using the in-house retention model. The alginate layer was demonstrated to be capable of supporting uncharged beads of up to 2 μm in diameter with no effect on the retention of the alginate dose. Charged particles greatly affected the retention of the alginate dose and appeared to be more adherent to œsophageal tissue in the absence of the alginate component.

This study demonstrated the feasibility of the alginate layer to act as a local drug delivery system targeted at the œsophagus.

7.2 SUGGESTIONS FOR FUTURE WORK

The aims of this project included the development of techniques used to assess the adhesion of a liquid formulation onto a biological substrate. This aim was achieved in the development of microscopic techniques used to assess the depth of the adhered alginate layer. However, an ideal technique would utilise staining procedures to distinguish between the alginate layer and the tissue surface. Such a system was attempted using PAS/Alcian blue stains on tissue-alginate sections as discussed in Chapter 3. The work was not reproducible and the results shown did not aid in the characterisation of the alginate adhesion. However, such a technique, if sufficiently developed would aid in the characterisation of alginate adhesion onto œsophageal tissue.

The preliminary study using confocal microscopy showed excellent results and provided an opportunity to visualise the alginate layer without any sample preparation. However, the resolution of the images was of low quality due to the low magnification objective lens used to gain the images. Instrumentation that provided the possibility of a large z range plus a high objective lens may be employed to investigate such a system and higher quality images may be observed. Such a system...
may also have the potential to assess an absolute value of the depth of the adhered layer rather than an indexed series of results.

Atomic force microscopy showed great potential in the characterisation of the alginate-oesophageal tissue interface. Unfortunately the results obtained using this technique did not provide the information that was hoped for. The technique may be greatly improved if sections of sufficient quality could be taken of a thickness well within the $z$ range of the probe. The large height difference between the tissue and alginate entities meant that the instrument was working at the limits of its capabilities and as such the results obtained were of poor quality.

Techniques including that described by Dobrozsi et al (1999) may be adapted for use in the characterisation of liquid alginate adhesion onto biological substrates. This technique used everted rat oesophagus and mass analysis to determine the retention of polymer solutions applied to the substrate. The method used physiologically relevant conditions to assess the retention of a liquid formulation onto a biological substrate. Such a method would aid in the characterisation of the systems investigated within this study. With hindsight a similar method would have added an extra dimension to the work as presented. However, this technique has disadvantages in the low sensitivity associated with the technique.

The work performed to analyse the mucin content of both saliva and oesophageal scrapings was performed on very small sample sizes. In retrospect this work would have been more creditable had larger populations been used. This fact stated the work was performed as a mini analysis to ensure that the conditions used were as physiologically "real" as possible and as such the conclusions made were not greatly affected by the small population sizes.

7.3 SUMMARY OF STUDY

The work in this study has provided new techniques with which the adhesion of a liquid formulation onto a biological substrate may be assessed. The results have proved that adhesion of an alginate solution on oesophageal tissue for time periods up to 60 minutes can be achieved. The ability of this adhesive layer to support model
drug particles has also been demonstrated. The techniques developed may be utilised to assess the retention of other liquid formulations to biological substrates. The oesophagus as a target organ has been investigated and the findings in this study may aid in the development of systems targeted specifically at the oesophagus.
APPENDIX I

Comparison of the viscosity values for the two artificial salivas used within the study. The viscosity was measured over a shear rate range from 0.1 – 10 s\(^{-1}\) at 37 °C. The viscosity of natural saliva is greater than the viscosity values for the two artificial salivas. However, the viscosity values of the two salivas are similar. Artificial saliva II has a higher mucin concentration and thus shows a higher viscosity value.

![Graph showing comparison of viscosity profiles](image)

**Figure A1.** Comparison of the viscosity profiles for the two artificial salivas
APPENDIX II

Comparison of the apparatus used in the retention model. The initial apparatus consisting of the Gallenkamp humidity chamber and the Perkin Elmer PE-204 spectrophotometer and the second set of apparatus were compared and their ability to assess the retention of an alginate dose is compared in Figure A2 below.

![Figure A2](image)

*Figure A2.* Comparison of the retention of an alginate dose using the two pieces of apparatus

Figure A2 above highlights the similarity in the retention profile of the alginate dose when assessed using the two different sets of apparatus. The above results were collected from a standard experimental run as decreed from the optimisation experiments.
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