INVESTIGATION OF LIQUID SODIUM ALGINATES AS MUCOADHESIVE BANDAGES COATING THE OESOPHAGEAL MUCOSA AND PROTECTING IT FROM GASTRIC REFLUX

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Summary

Sodium alginate is a natural unbranched polysaccharide extracted from seaweed and composed of two basic units; β-(1, 4)-linked D-mannuronic acid (M unit) and α-(1, 4)-linked L-guluronic acid (G unit) residues. Because of their stable physico-chemical properties alginates are used in a variety of aspects in many industries, including pharmaceutical, medical and food.

Gastro-oesophageal Reflux Disease (GORD), is generally caused by excess gastric reflux back to the oesophagus where damage to the mucosa results in injury. GORD is a very common disease in western countries, more than a quarter of western people are suffering from this disease and there is a trend that the percentage population in eastern countries who are diagnosed as GORD is increasing. GORD and its complications damage the quality of life and can lead to serious oesophageal diseases including Barrett's disease and oesophageal carcinoma.

Sodium alginate dissolved in water forms a viscous liquid and can coat on oesophageal mucosa for a period of time. In this study the ability of the liquid alginate to adhere to the oesophageal mucosa was investigated and the factors that affect this retention were examined. The potential of this liquid alginate as a drug delivery vehicle to extend the duration of contact with the oesophageal mucosa was confirmed by this study. The capacity of an alginate coating to retard acid and pepsin diffusion, the two main aggressive factors in gastric reflux, was investigated. A significant reduction in acid and pepsin diffusion by alginate gel layer was demonstrated in this project, indicating that alginate has great potential to protect against damage caused by acidic reflux. A novel method was introduced using an independent score system to assess the protection of oesophageal tissue by a coating of liquid alginate using microscopy as a technique. This technique demonstrated that alginate can protect the oesophageal epithelial tissue from the damage caused by gastric acid and pepsin.

Many techniques were used in this study. The experimental results suggested that liquid sodium alginate is a very promising candidate in treating local oesophageal diseases through forming a coating on the oesophageal mucosal surface, retarding the diffusion of components of gastric refluxate and thus reducing the contact of these noxious factors with the epithelium and minimising injury.

***Key Words*:** Sodium alginate, mucoadhesive, gastric reflux, acid, pepsin, oesophagus.

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ABBREVIATIONS

ADI	Acceptable Daily Intake
AUC	Area Under the Curve
DMED	Dulbecco's modified eagle medium
DMF	Dimethyl Formamide
EDAC	1-ethyl-3(3-dimethylaminopropyl) carbodiimide
EGF	Epidermal Growth Factor
FCS	Foetal calf/bovine serum
GORD	Gastro-oesophageal Reflux Disease
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOS	Lower Oesophageal Sphincter
MEM	Minimum essential medium
MWCO	Molecular Weight Cut Off
PGA	Propylene Glycol Alginates
PGE_2	Prostaglandin E_2
TLOSR	Transient Lower Oesophageal Sphincter Relaxation

CHAPTER 1 INTRODUCTION

1. 1 THE OESOPHAGUS

The oesophagus serves to move food boluses, drink or drug formulations from the oral cavity through the oesophagus and lower oesophageal sphincter (LOS), into the stomach. In healthy people, ingested materials have a very short transit time through the oesophagus, but this is slightly lengthened when individuals are supine due to the loss of gravitational forces. Another function of the oesophagus is control of physiological reflux. The lower oesophageal sphincter (LOS) closes to reduce the volume of gastric reflux and is involved in the secondary peristaltic wave that clears the retained refluxate from within the oesophageal lumen to maintain a neutral environment.

1.1.1 Anatomy and physiology of the oesophagus

The oesophagus is a muscular tube, located behind the trachea and heart, in front of the spinal column. It passes through the chest diaphragm and enters the stomach. The average length of oesophagus is 23 cm with a range from 17-30 cm (Li et al., 1994) and 1.5-2 cm diameter (Mercer & Hill, 1988). The anatomical structure of the oesophagus consists of the upper oesophageal sphincter, lower oesophageal sphincter and the oesophageal muscular body.

The upper oesophageal sphincter is located at the meeting of pharynx and oesophagus. The sphincter is striated muscle forming a ring that acts to open and close during swallowing. It is in a state of tonic contraction with relaxation mediated from vagal stimulation. This relaxation and contraction of the upper sphincter is caused by the stimulation of food swallowing initiating primary peristalsis to propel food boluses down the oesophageal tube (Lang & Shaker, 1997).

The lower oesophageal sphincter (LOS) represents the transition between the oesophagus and stomach and is about 3-4 cm in length (Sloan et al, 1992). It is at the junction of squamous and columnar epithelium and at this point the oesophagus meets the stomach. The LOS is regarded as the major component of the anti-reflux barrier. The sphincter muscle is thicker than that of the adjacent oesophagus (Liu et al, 1992), mainly due to tonic contraction (Liu et al, 1997). The LOS maintains a normal resting pressure that has an important role in the anti-reflux barrier.

The oesophageal body, between the upper and lower oesophageal sphincters, serves to transit swallowed boluses to the stomach. The resting pressure of the oesophagus changes due to breathing. During inspiration the pressure is between -5 to -10 mmHg and during expiration it is 0 to 5 mmHg (Washington et al, 2001). The swallow is associated with a transient decrease in oesophageal pressure followed by a primary peristaltic wave of high pressure which travels towards the stomach at a speed of 2 to 6 cm/s in the proximal oesophagus and gradually becoming faster to the distal portion (Washington et al, 2001). The average speed of this peristaltic wave is 3.0 – 3.5 cm/s (Humphries & Castell, 1977) and each wave takes about 7-10 seconds. Oesophageal luminal acidification and bolus distension initiate secondary peristalsis for further clearance of the residue left in the oesophageal lumen.

There are four layers within the oesophagus (Figure 1.1); a fibrous external layer, a muscular layer, a submucous layer and an internal mucous-type layer (Washington et al., 2001). The external fibrous layer consists of elastic fibres. The muscular layer is composed of circular muscle surrounded by longitudinal muscle. The lower third of the oesophagus is smooth muscle, striated muscle in the upper part and both types are found in the middle section. The submucous layer contains larger blood vessels, nerves and mucous glands that loosely connect the mucous and muscular layers. The internal mucosal layer is covered with a layer of stratified squamous epithelium lining extending from the buccal cavity continuing through the pharynx and down to the oesophagus and ends at the LOS. It provides a tough impermeable lining resisting the abrasive nature of food boluses.

Figure 1.1 Cross-section schematic diagram of the human oesophagus **(Leeson et al, 1988)**

The oesophageal glands are located in submucosa and distributed throughout the length of the oesophagus, they are small racemose glands of mucus type and each with a long duct open to lumen (Washington et al, 2001). About 600 – 700 glands in total are present in the oesophagus making the oesophagus moist rather than wet (Namiot et al, 1994a). The main function of the secretions is to lubricate the tube and protect the lower part of the oesophagus from gastric reflux via the pre-epithelial defensive layer of the oesophagus.

1.2 OESOPHAGEAL DISEASES

1.2.1 Oesophageal Diseases

1.2.1.1 Gastro-oesophageal reflux disease (GORD)

Gastro-oesophageal reflux disease (GORD) is characterised by increased exposure of oesophageal mucosa to gastric contents. This is mainly due to the disequilibrium between mucosal protective mechanisms and increased attack from aggressive factors, leading to a variable combination of an increased number of gastro-oesophageal reflux episodes and abnormally prolonged clearance of the refluxed materials (Orlando, 2000).

Gastro-oesophageal reflux disease is a common disease in western countries. The main symptoms include heartburn and regurgitation. Heartburn is described as "a burning feeling rising from the stomach or lower chest up towards the neck" (Dent et al, 1998) and this symptom is likely relate to certain activities, like after a heavy meal, bending over, lifting and lying down. Regurgitation is a feeling of ingested material backing up

to the throat. Persistent, serious symptoms can seriously hamper the quality of a patient's life (Locker et al, 1997). GORD greatly increases the likelihood of developing other more serious oesophageal diseases and complications, such as oesophageal stricture, dysphasia, Barrett's oesophagus and oesophageal carcinoma due to persistent tissue damage (Lagergren et al, 1999).

1.2.1.2 Barrett's oesophagus and oesophageal cancer

Barrett's oesophagus is defined as a true glandular metaplasia of the lower oesophagus. Acid reflux remains the prime candidate for causation of this disease (Coad & Shepherd, 2003). There is considerable evidence that has confirmed the importance of intestinal metaplasia (columnar epithelial cells replace the squamous epithelial cells lining the lower oesophageal mucosa) in neoplastic transformation in Barrett's oesophagus, which increases a patient's risk of oesophageal adenocarcinoma by 30-40 %, and the prognosis for such patients is poor (Van der Burgh et al, 1996). Patients with Barrett's oesophagus develop oesophageal adenocarcinoma at the rate of approximately 0.5 % per year (Shaheen et al, 2000).

1.2.1.3 Other local oesophageal diseases

Achalasia is an oesophageal motor disorder with unknown aetiology, resulting from insufficient LOS relaxation and ineffective peristalsis. The main symptoms of achalasia are dysphagia and regurgitation of ingested food and drink. Currently there is no cure for this problem. Nitrites and calcium channel blockers are used in the pharmacological management as these drugs relax the smooth muscle of the oesophagus, thus allowing the passage of an ingested bolus. Infections of the

oesophagus, for example oesophageal *Candida*, are primarily associated with immuno-compromised hosts, including patients undergoing cancer chemotherapy or those with HIV (Moorman, 1997).

1.2.2 Diagnostic methods

For GORD, heartburn frequency is an important facet upon which the diagnostic evaluation must be based and reflux disease is likely to be present when heartburn occurs on two or more days a week (Dent et al, 1998). Other historical features of GORD are reflux symptoms that occur predominantly after food intake; in the absence of peptic ulcer or oesophagitis; upper abdominal/lower chest symptoms are relieved by antacids; it is unusual for reflux symptoms to disturb sleep. Diagnosis of other oesophageal diseases and complications are dependent upon barium oesophageal, endoscopy and biopsy that is a "golden standard" for diagnosis of Barrett's oesophagus and carcinoma.

1.2.2.1 Barium oesophagography

Barium oesophagography (also known as barium swallow) has greatest utility in detecting the complications of GORD rather than demonstrating reflux, by showing erosive oesophagitis and oesophageal strictures. It also helps to identify other anatomical problems such as pyloric stenosis or intestinal obstructions. But has major limitations in evaluating reflux.

1.2.2.2 Endoscopy

Endoscope examination is more accurate than a barium-swallow radiograph, and should therefore be the initial evaluation technique because it provides the most sensitive method for detecting oesophageal manifestations. Although endoscopy is commonly used for screening Barrett's/adenocarcinoma to control the risk of cancer, it is also encouraged in patients suffering from dysphagia, or gastrointestinal bleeding (Dent et al, 1998; DeVault & Castell, 1999). Oesophageal endoscopy is currently the "gold standard" for the gastroenterologist in diagnosing oesophageal diseases. If a stricture is identified during endoscopy, therapy can be rendered during the same patient encounter. That means endoscopy can be used in both diagnosis and treatment.

1.2.2.3 Ambulatory oesophageal pH monitoring

24 hours oesophageal pH monitoring is very popular as an objective method of estimating the presence of excessive gastro-oesophageal reflux. The most common measures include total reflux time, the number of reflux episodes, and the number of episodes lasting longer than five minutes. Although some centres reported reducing the length of testing has given similar patterns of reflux in symptomatic patients and control subjects (Ogorek, 1995), the 24 hours ambulatory pH monitor is still the standard for reflux qualification. However, an oesophageal catheter positioned in the oesophageal tube for 24 hours is associated with discomfort and inconvenience to patients.

1.2.3 Treatment of oesophageal diseases

Because GORD is one of the most common oesophageal diseases and it increases the possibility of developing other more serious oesophageal diseases, effective treatment of GORD is very important. Generally it includes lifestyle modification, acid suppressive therapy, and prokinetic agents. If more serious problems occur, like Barrett's oesophagus or carcinoma, then surgical treatment may be introduced.

1.2.3.1 Lifestyle modification

Although there is little evidence to support a significant role of lifestyle factors as a major cause of GORD, lifestyle modification is still commonly recommended in clinical practice guidelines (DeVault & Castell, 1999). Lifestyle modification includes: avoidance of tight fitting garments, to reduce stomach pressure; restriction of some foods, especially alcohol since alcohol directly irritates the oesophageal mucosa and reduces LOS pressure, also high fat foods can decrease the LOS pressure; smoking cessation can significantly reduce reflux frequency, improve the mucosal barrier quality and recover the salivary secretion rate; weight loss and avoiding a supine posture after meals also helps to reduce reflux.

1.2.3.2 Acid suppression therapy

The most common and effective treatment of oesophagitis is to reduce the acid secretion. H_2-receptor antagonists and proton pump inhibitors (PPIs) are the two main medicines used to suppress acid secretion.

Four H_2-receptor antagonists are currently used in clinical treatment within the UK, cimetidine, ranitidine, famotidine and nizatidine. These agents bind to the H_2 receptor on the gastric parietal cells resulting in inhibition of basal and stimulated acid secretion. Five PPIs are currently in clinical use; omeprazole, lansoprazole, pantoprazole, rabeprazole and esoprazole. The mechanism of PPIs to control the symptoms and heal lesions in patients with GORD is by superior inhibition of gastric acid secretion through the blockade of the parietal cell's final common pathway for acid secretion, the enzyme H^+/K^+ ATPase ("proton pump") (Wallmark, 1986). The effective treatment periods of these two agents in healing oesophagitis are 6-12 weeks; H_2 antagonists offer a therapeutic gain of 10-24 % relative to placebo for healing oesophagitis, however PPIs gain more efficacious healing result, exhibiting 57-74 % therapy in relative to placebo and achieving more than 90 % healing overall (Kahrilas, 2000). PPIs are also used as first-line therapy to relieve the symptoms of Barrett's oesophagus (Klinkenberg-Knol et al, 2000).

1.2.3.3 Prokinetic agents

Prokinetic drugs are used in the treatment of GORD by counteracting some of the associated physiological abnormalities including; increasing LOS pressure; enhancing gastric emptying; or improving peristalsis. But the considerable side effects of these medicines on the central nervous system limit their use, for example, bethanechol has the cholinergic effect to increase gastric acid secretion, cause bronchoconstriction and bladder contraction. Metoclopramide has a 20-50 % incidence of fatigue, tremor, Parkinsonism (Miller & Jankovic, 1989). So prokinetic agents are not common agents used in the treatment of GORD.

1.2.3.4 Endoscopic treatment

Lifestyle modification sometimes is disagreeable for the patient, and long-term medication is not acceptable for some patients, therefore endoscopic treatment can be a good choice. Patients with complicated GORD including Barrett's oesophagus, stricture or carcinoma are not suitable candidates for endoscopic treatment. The methods of endoscopic treatment for GORD are either to tighten the region of LOS by sutures or clips, cause collagenisation at the gastro-oesophageal junction by augmenting the junction by injecting substances into the tissue of gastro-oesophageal junction (Dhawan, 2002). All of these treatments result in an increase in the barrier function of the LOS to minimise the volume of gastric reflux.

1.2.3.5 Surgical treatment

When serious problems occur, such as Barrett's oesophagus, oesophageal adenocarcinoma, surgical management is generally used to remove the damaged tissue. In the absence of overt complications of GORD, failure of medical therapy to control the symptoms after a medical trial of at least 6 – 12 months could also be a reason for undertaking surgical treatment (Grant, 1999).

1.3 MECHANISMS OF OESOPHAGEAL DAMAGE

1.3.1 Pathophysiological mechanisms of oesophageal epithelial injury

In humans, the oesophageal epithelium has considerable capacity to resist damage even from direct contact by high concentrations of luminal acid. However, when

luminal acidity is sufficiently noxious, tissue resistance can be overcome, leading to heartburn and ultimately cell necrosis (Orlando, 2000).

The studies of Khalbuss et al. (1995) indicated that at pH 2 or greater, the structural barriers (cell membrane, intercellular junctional complex) play the major role against luminal acid injury. At pH less than 2.0, the structural barrier alone is not enough, gravity and oesophageal peristalsis clear most of the acid and pepsin, bicarbonate buffer produced by salivary and oesophageal submucosal glands neutralise the acid. HCO_3^- within the intercellular spaces is very important in buffering H^+ (Tobey et al, 1989). The buffering capacity of the cells and intercellular spaces in part, depend on the blood supply (Christie et al, 1997). If the buffering capacity is overcome, the oesophageal cell has to remove the proton by two mechanisms; Na^+/H^+ exchange and Na^+ dependent Cl^-/HCO_3^- exchange (Tobey et al, 1993). If the luminal acidity persists, the cells are continuously acidified and a pathological change in cells will occur.

High luminal acidity attacks causing damage to the intercellular junctional complex was proven in the studies by Orlando et al (1981; 1984) and Tobey et al (1991) showed that epithelial permeability increased with acid injury as the earliest change. A morphologic change due to acid injury was demonstrated by the presence of dilated intercellular spaces within the oesophageal epithelium (Tobey et al, 1997). As intercellular junctions guard the intercellular space, protons must enter the intercellular spaces of oesophageal epithelium through the paracellular pathway. Acidification of the intercellular spaces leads to two consequences. One is ready access of acid to the sensory nerve endings within the epithelium, which are located in the intercellular space only a few cell layers below the luminal surface (Rodrigo et al, 1975). This may explain why patients with non-erosive oesophagitis complain of heartburn. Secondly,

acidification of intercellular space permits H^+ to enter the basolateral membrane of the epithelial cell. Basolateral membranes are more permeable to H^+ ions than apical membranes (Christie et al, 1997). Oesophageal cells are therefore very sensitive to the basolateral acidification that causes cell necrosis.

Evidence suggested that acid initiates cell damage by increasing intracellular calcium that activates a basolateral membrane, NaK_2Cl co-transporter (Tobey et al, 1997). At low pH the NaK_2Cl co-transporter brings ions and water into the cell, the latter serves to increase pH. At acid pH, extrusion of excess ions by the NaK ATPase transporter is inhibited. This results in loss of osmo-regulation, cell swelling and oedema, leading to cell death by cell membrane rupture. Another mechanism for cell death is the damaged cells release inflammatory mediators, which in turn, recruit white blood cells to the injury area, which serves as another means of cell damage and necrosis (Naya et al, 1997).

1.3.2 Causes of oesophageal mucosa injury

In gastric refluxate there is not only acid, but also other components, such as pepsin, bile acid and trypsin present in the gastric contents. Acid is a well-known factor in injury of the oesophageal epithelium. The activity of pepsin is limited to pH values below 3, but it produced an irreversible and more serious lesion on epithelium than acid alone (Tobey et al, 2001). Although many animal studies demonstrated that bile and trypsin can cause severe oesophageal lesions (Lillemoe et al 1983; Salo et al, 1988), there is no correlation between bile acid concentration and the grade of oesophagitis (Stoker & Williams, 1991). In addition, trypsin was rarely found in

gastric refluxate, so bile and trypsin are probably less important in reflux oesophagitis (Gotley et al 1991).

Gastric reflux occurs in all people, yet it is only when this refluxate is retained in the oesophageal lumen for excessive periods that tissue damage occurs. Many reasons cause delayed oesophageal emptying, such as malfunction of LOS, hiatus hernia and dysfunction of oesophageal motility.

As the LOS is the main component of the anti-reflux barrier, dysfunction of the LOS is the main mechanism at fault in gastro-oesophageal reflux. The weakness of either the LOS or crural diaphragm or both, play a role in severe oesophagitis. A large study indicated that transient lower oesophageal sphincter relaxation (TLOSR) is the major mechanism accounting for 60-80 % reflux episodes (Dodds et al., 1982; Dent et al, 1988a). TLOSR are defined as LOS relaxations not triggered by swallowing. They are of longer duration (10-45 s) than swallow-induced LOS relaxation (5-8 s) (Mittal & Balaban, 1997). Low pressure could potentially predispose to reflux during straining, particularly when LOS function is further compromised by the presence of a hiatus hernia (Sloan et al, 1992).

Hiatus is a small hole in the diaphragm through which the oesophagus passes into the stomach. Displacement of the LOS from the diaphragmatic hiatus into the chest is associated with a reduction in basal LOS pressure (Kahrilas et al, 1995a). Kahrilas and his colleagues (2000) also suggested that hiatus hernia may increase the triggering of TLOSR. The clearance of refluxate is delayed by hiatus hernia because the increased tendency of reflux retained within the hernial sac (Chana, 1996). All of the above mechanisms contribute to the increasing of occurrence of GORD.

Many other mechanisms cause reflux diseases. Ineffective oesophageal body peristalsis delays the clearance of the gastric contents retained within the oesophagus (Kahrilas et al, 1986). However Simrén et al (2003) suggested ineffective oesophageal motility had little effect on delayed oesophageal clearance during upright positions and only severe ineffective oesophageal motility may prolong the clearance. At least half of asthmatic patients have GORD that may be triggered by coughing and sneezing accompanying asthmatic attacks causing changes in chest pressure. Impaired stomach function like stomach muscle unable to contract normally thereby causing a delay in gastric emptying, increases the risk of acid reflux. A twin study done by Mohammed et al (2003) indicated that genes may have some influence in GORD. Oesophageal injury and dysphasia can be drug-induced (O'Neill & Remington; 2003), especially in elderly patients, patients with pre-existing oesophageal disorders and HIV/AIDS patients.

People of all ages are susceptible to GORD. Anyone who eats a heavy meal, then lies on their back or bends over is at risk of heartburn. Pregnant women are particularly vulnerable to heartburn as the growing uterus increases the pressure on the stomach. People with obesity are at higher risk to experience GORD (Stene-Larsen et al, 1988). Smoking and alcohol relax the LOS muscle and increase stomach acid levels (Kahrilas et al, 1995b), leading to a greater chance of GORD. Smoking prolongs acid clearance by reducing the salivary secretions, therefore also limiting the neutralising component from swallowed saliva (Kahrilas & Gupta 1989).

1.4 MECHANISMS OF OESOPHAGEAL MUCOSAL PROTECTION

The oesophageal mucosa is exposed to exogenous substances, such as food, drink and drug formulations, and also to endogenous materials, like refluxed acid, pepsin and bile salts as well. The integrity of the oesophageal mucosa depends on the equilibrium between aggressive factors and protective mechanisms. The oesophagus lacks many mechanisms exhibited by the stomach to protect the epithelium against refluxed gastric contents, but many mechanisms maintained by the oesophagus help against acid damage; lower oesophageal sphincter (LOS) pressure, the motility of oesophagus and four epithelial related defensive mechanisms are described in detail below. The mechanism of epithelium defence is also called mucosal defence and is divided into four parts; pre-epithelium, epithelium, post-epithelium and tissue repair mechanisms (Yoshida & Yoshikawa, 2003).

1.4.1 Pre-epithelial defensive mechanisms

Pre-epithelial defence is a mucus-buffer layer covering the oesophageal mucosa, the thickness of which is approximately 95 ± 12 μm (Sarosiek & McCallum, 1995). This layer is comprised of salivary and oesophageal components.

Saliva is a very important element contributing to the oesophageal pre-epithelial defence. Human saliva is secreted by three pairs of major salivary glands, the parotid, the submandibular, the sublingual glands and some minor salivary glands in the oral cavity as well. Olfactory sense and taste of food potently stimulate an increase in the quantity and quality of salivary secretions (Hightower, 1966). Masticatory stimulation is able to enhance the salivary secretion in both inorganic components (bicarbonate

and non-bicarbonate buffers) and organic components (salivary non-mucin proteins, mucins, EGF, TGFα and PGE_2) (Sarosiek et al, 1996). Basal secretion of saliva ranges from 0.4 to 0.6 mL/min with a HCO_3^- concentration of 4 mM (Richardson et al 1986). Acid and pepsin evoke an enhancement of bicarbonate, prostaglandin and Epidermal Growth Factor (EGF) output (Namiot et al, 1994b; Namiot et al, 1997; Li et al, 1993). Bicarbonate and non-bicarbonate buffers neutralise the acid, which in turn inactivates the pepsins as the pH increases (Tobey et al, 2001). Prostaglandins have an ability to improve the viscosity of mucus and retard hydrogen ion diffusion and inhibit pepsin penetration from the lumen into the mucosa, whereas EGF enhances stem cell proliferation (Sarosiek et al, 1986) that improves the repair of damaged epithelium. Some studies have shown the existence of EGF receptors on the cellular membrane within the oesophageal mucosa (Gray et al, 1993; Jankowski et al, 1992).

It is not only salivary secretions that significantly contribute to pre-epithelium defences, but also oesophageal mucus has an important role in mucosal protection. The human oesophageal mucosa contains numerous submucosa mucous glands (Namiot et al, 1994b). These glands can not only secrete bicarbonate and non-bicarbonate buffers, but also mucin, non-mucin proteins, EGF, TGRα and prostaglandin E_2 (Sarosiek et al, 1994; Meyers & Orlando, 1992). Exposure of the oesophageal lumen to acid and pepsin results in an increase in release of inorganic and organic components of the oesophagus (Sarosiek et al, 1994).

1.4.2 Epithelial defensive mechanisms

If the pre-epithelial mucus-buffer layer can not inactivate the injurious components, it will be eroded by the proteolytic activity of acid and pepsin along with the detergent-

like activity of bile acids, thereby leading to the exposure of epithelial surface to the aggressive factors. In fact the oesophageal pre-epithelium defence has a very limited ability to prevent luminal acidity from reaching the epithelial cell surface. The work done by Quigley and Turnberg (1987) showed that perfusion of the oesophageal lumen with pH 2.0 results in a surface pH of 2.0 to 3.0. Epithelial defence has to undertake the bulk of battle against the injury by gastric materials.

The oesophageal mucosa is capable of significantly retarding hydrogen ion diffusion and completely stopping the diffusion of pepsin and bile components. Two mechanisms contribute to this defence. Firstly, morphological components participate in the defence; cell proliferation and migration replace the lost cells due to cell injury; the hydrophobic phospholipid bi-layer of the cell membrane; tight junctions; intercellular lipid and glycoprotein structures all contribute to the quality of the epithelial barrier (Orlando et al, 1992; Tobey & Orlando, 1991). If these morphological barriers are not able to stop hydrogen ion diffusion, intracellular systems, such as intracellular buffers, Na^+/H^+ exchange and Na^+-dependent Cl^-/HCO_3^- exchange may eliminate some of the H^+ and thereby maintain the homeostasis of H^+ in intracellular systems to maintain the integrity of the oesophageal mucosa (Orlando, 1991)

1.4.3 Post-epithelial defensive mechanisms

The basement membrane, oesophageal mucosal vasculature and its blood flow actively participate in the post-epithelial protective mechanisms. Early work done by Hollwarth et al (1986) reported that oesophageal blood flow of the cat was markedly increased when the oesophagus was exposed to injurious substances, such as acid, pepsin and

bile for a short time. It is considered that oxygen and bicarbonate from the microcirculation of oesophagus remove the hydrogen ions that diffuse from the intracellular spaces. However, exposure of the oesophagus to these injurious materials for a long time, resulted in suppression of the blood flow and mucosal damage (Bass et al, 1984).

1.4.4 Tissue repair mechanisms

The tissue repair function not only acts when oesophageal defensive mechanisms are overcome, but also occurs when the aggressive factors contact the oesophageal mucosa. As stated in the pre-epithelium protective mechanisms, when the oesophageal mucosa is exposed to gastro-duodenal refluxate, salivary and oesophageal submucosal glands respond to greatly increase the secretion of inorganic and organic components. Among them HCO_3^- neutralises H^+, mucin reduces proton diffusion, prostaglandin E_2 increases the viscosity of mucin thereby further retarding the diffusion of hydrogen ions, epidermal growth factor (EGF) stimulates DNA synthesis to improve cell proliferation and nitric oxide maintains the oesophageal microcirculation to assist with mucosal protection (Yoshida & Yoshikawa, 2003). Tissue repair processes exist as long as the aggressive factors attack the mucosa.

1.4.5 The role of the LOS and secondary peristalsis

The LOS plays a very important role in the anti-reflux barrier. The normal resting pressure is at a range of 10-30 mmHg relative to intra-gastric pressure. But usually the minimal basal resting pressure of the LOS is in the range of 5-10 mmHg and can prevent gastric reflux (Dent et al 1988b). The basal LOS pressure varies at different

times; it is lowest after a meal and highest at night. The pressure increases in response to an increase in intra-abdominal pressure and such gastro-oesophageal junction pressure increase is due to not only LOS tone increasing but also the extrinsic compression by the crural diaphragm (Mittal et al, 1990). This activity is very important in preventing reflux during vigorous gastric contractions. The crural diaphragm is another important factor that contributes to the resting pressure by squeezing the intrinsic oesophagus and supporting the LOS segment during straining. The LOS pressure is mediated by cholinergic nerves since basal pressure can be reduced by up to 70 % with atropine (Dodds et al, 1981).

Secondary peristalsis is initiated by distal irritated gastric reflux. This activity is extremely effective at removing the overall volume of refluxate (Williams et al, 1993). For instance, perfusion of the distal oesophagus with 15 mL acid initiated a secondary peristaltic wave that reduced the volume to 1 mL within 15 seconds (Helm, 1984). Thompson et al (1988) suggested that secondary peristalsis was generated by the response of the oesophagus to bolus distension rather than to refluxed acid.

1.4.6 Comparison of oesophageal and gastric epithelial structures

Oesophageal mucus comes from two sources; one is the refluxed gastric mucus that is secreted from goblet cells, another source is from swallowed salivary mucus. Given the fact that moderate degrees of acid suppression can effectively treat gastric peptic ulcer disease, yet are limited in healing oesophagitis and most patients with reflux disease have normal rates of acid and pepsin secretion (Hirschowitz, 1991). There are four known differences in tissue resistance between the oesophagus and stomach (Orlando, 1991), which are presented in Figure 1.2.

The first is the difference in the construction of the pre-epithelial defence that consists of mucus and an unstirred water layer, the latter rich in bicarbonate ions. In the stomach, bicarbonate ions are principally secreted by the surface epithelial cells via paracellular diffusion from interstitial fluid across tight junctions to the epithelial surface. This pre-epithelial defence is capable of maintaining the pH at the epithelial cell surface at pH 5-7 even though the pH of the luminal contents of the stomach are as low as pH 1-2 (Quigley & Turnberg, 1987). On the contrary oesophageal pre-epithelial defences are a poorly developed unstirred layer, where small amounts of mucoprotein are present. The unstirred water layer can not hold HCO_3^- secreted from oesophageal glands because it is too thin (Yoshida & Yoshikawa, 2003), a less leaky paracellular route retards bicarbonate diffusion from interstitial fluid to the luminal surface. In the human oesophagus, the pre-epithelial layer can only maintain a gradient of one pH unit from lumen to apical cell surface (Quigley & Turnberg, 1987).

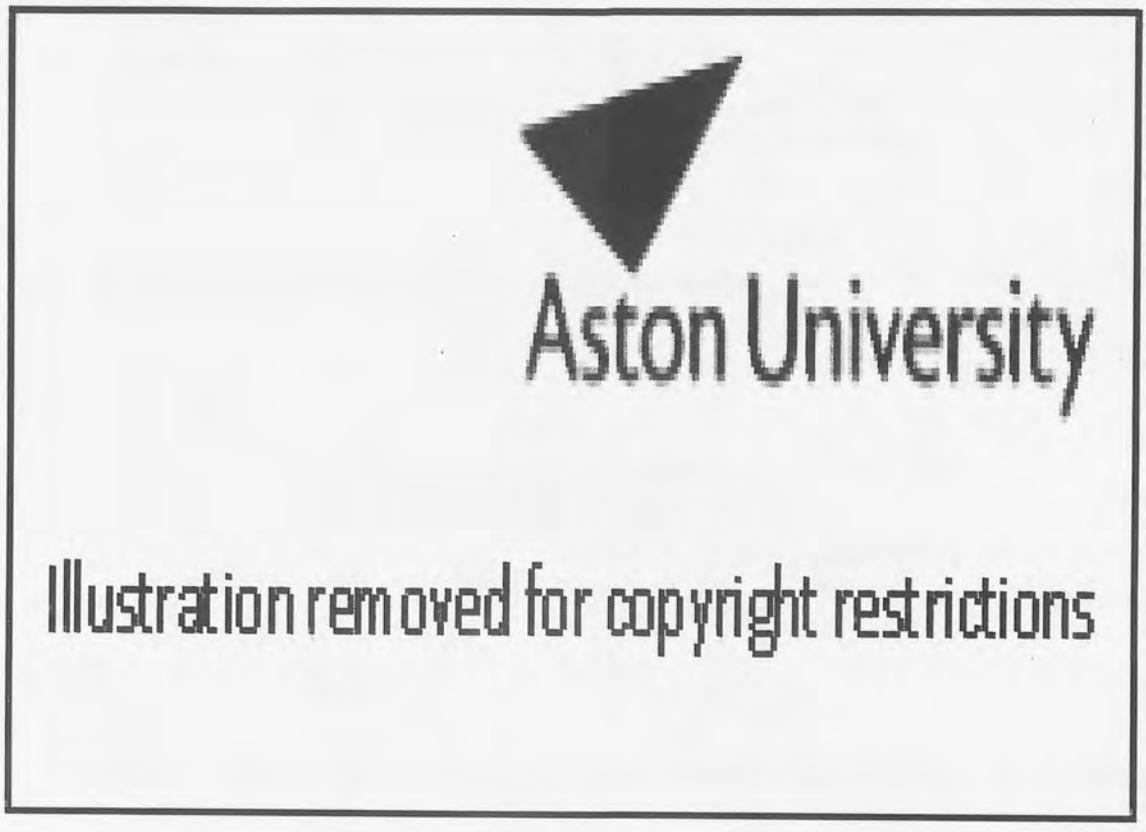
The second difference between the oesophageal and gastric epithelium is the tissue response to contact with irritant substances. When exposed to acid, the columnar epithelium of the stomach responds by release of prostaglandins, which in turn enhance epithelial protection of the tissue by stimulating the secretion of mucus and bicarbonate and increasing or stabilising mucosal blood flow (Morris et al, 1990). However, although prostaglandins are also released when the oesophageal epithelium is exposed to acid, it appears to be devoid of increasing epithelial protection by inability of oesophageal cells to respond to prostaglandins by secretion of mucus and bicarbonate (Sarosiek et al, 1994).

The ability of rapid repair is a third difference between oesophageal and gastric epithelium. After an acute injury by acid or other noxious elements gastric epithelium

is capable of repairing itself in 30-60 minutes, a process that is termed "epithelial restitution" (Silen, 1987). Epithelial restitution occurs rapidly because it does not require cell replication but uses its ability of viable neighbouring cells to migrate over a bare basement membrane until it contacts with its counterpart from the other side of the wound. This restores the integrity of the epithelial surface and makes it less vulnerable to further acid attack. But epithelial restitution does not occur in the oesophagus because the injured surface area is far from the basement membrane (Tobey et al, 1989), which means that after injury, repair of oesophageal epithelium depends on cell replication that takes days to weeks. During this period the breach in the epithelial barrier may be exposed to acid, pepsin and bile that damage the deeper regions of the tissue.

A fourth difference is the development of a so-called "mucous cap" (Silen, 1987) that occurs after tissue injury and forms over the injury area in the stomach, but not the oesophageal epithelium. It consists of epithelial cell fragments, inflammatory cells including red and white blood cells, plasma proteins and plasma bicarbonate by so doing to provide an environment of pH-neutral or near-neutral pH that enables tissue repair. In the absence of such a mucous cap, luminal acid easily accesses the damaged region and inhibits the cell reparative function as in the case of oesophagus.

Figure1.2 presents the difference of pre-epithelial defence between the oesophagus and stomach.



The weak pre-epithelium defences; lack of epithelial cell ability to respond to prostaglandin release with mucus and bicarbonate secretion; the lack of mucous cap to create a neutral environment to permit repair and lack of epithelial restitution to seal the injury area, all of the above statements make the oesophageal epithelium readily accessible to luminal acid and pepsin after injury and difficult to repair. The latter three points can not be improved as the oesophagus itself lacks those physiological protective mechanisms. So improving the oesophageal defence could focus on two aspects; increasing pre-epithelium defences and oesophageal luminal pH by reducing

the quantity of acidic gastric contents in the oesophageal lumen and shortening the duration of gastric refluxate retained on the oesophageal mucosa. Table 1.1 summarises the differences in tissue resistance between the oesophagus and the stomach

Adhesion is defined as the state in which two surfaces are held together by interfacial forces. If one or both of the surfaces are of a biological nature, adhesion is referred to as *bioadhesion*, the term *mucoadhesive* is defined as a process where synthetic and natural macromolecules adhere to the mucosal surface in the body. If these materials are incorporated into pharmaceutical formulations, drug absorption by mucosal cells may be enhanced due to drug release at the site for an extended period of time (Woodley, 2001). Most frequently, bioadhesive drug delivery systems operate in an

environment in which a mucus gel layer acts as an interface between adherent and epithelial surfaces.

1.5.1 Mechanisms and theories of bioadhesion

There are no complete and clear mechanisms and/or theories to explain the process of bioadhesion. The processes involved in the formation of bioadhesive bonds can be described in three steps: first, wetting and swelling of polymer to permit intimate contact with biological tissue; second, interpenetration and entanglement of bioadhesive polymer chains with mucin chains; and third, formation of weak chemical bonds between entangled chains (Duchêne et al, 1988). In general, five theories have been adapted to the study of bioadhesion: the electrostatic theory; the adsorption theory; the diffusion theory; the wetting theory and the fracture theory.

The *electrostatic theory* assumes that when the two materials contact with each other, electron transfer across the interface leads to the formation of a double layer of electrical charge and subsequently, a system of attractive forces that maintain the contact (Derjaguin & Smilga, 1969). The *adsorption theory* states that the adherence between adhesive substrate and tissue or mucosa is due to van der Waals interactions or hydrogen bonds. Although these forces are weak, the sheer number of interactions can produce intense adhesive strength (Kinloch, 1980). The *interpenetration or diffusion theory* is perhaps the most widely accepted physical theory and the most relevant to bioadhesive hydrogel systems. In the mucoadhesive context, polymeric chains, from both the bioadhesive and mucus, intermingle and reach a sufficient depth within the opposite matrix to allow formation of a semi-permanent bond. The depth of penetration depends on their diffusion coefficient that is associated with the molecular

weight of the polymer (Reinhart & Peppas, 1984). Once intimate contact occurs, the bioadhesive polymeric chains will be driven to move along their concentration gradients into the opposing phase until an equilibrium penetration depth is achieved. In the context of bioadhesive drug delivery systems, a defined residence time is required at the target site, after which they are removed or rendered inactive. *The wetting theory* was developed with regard to liquid adhesives and uses interfacial tensions to predict spreading and in turn adhesion (Baszkin et al, 1990). *The fracture theory* is the most measurable theory that analyses the forces required to separate two surfaces after adhesion (Peppas et al, 1987). But one critical assumption of this theory is that the investigated system has to exhibit known physical dimensions and be composed of a single uniform bulk material, this cannot be guaranteed.

1.5.2 Sites for mucoadhesive drug delivery systems

In the human body all sites covered with mucosal membrane could be chosen as bioadhesive targets. So the buccal cavity, vagina, nasal cavity, eyes and GI tract are principal places for mucoadhesive targeting.

The buccal cavity has a relatively small surface area, approximately 50 cm^2 (Lee et al, 2000), but some advantages make it a useful site, such as no first-pass metabolism and non-keratinised epithelium that is relatively permeable to drugs. Some studies have achieved good results when a drug within mucoadhesive was applied on buccal mucosa. Agarwal & Mishra (1999) demonstrated that buccoadhesive compacts of carbopol and hydroxypropyl methyl cellulose containing pentazocine produced a 2 to 3-fold increase in the bioavailability when administered to rabbits compared with conventional oral administration. The vagina is a highly suitable site for bioadhesive

formulations; some bioadhesive formulations have been successfully brought to the market (Woodley, 2001). Crinone ®, produced by Columbia laboratories is a product for treating problems associated with infertility, uses adhesive technology to ensure a controlled release of progesterone for at least 48 hours after a single vaginal application. The nasal cavity is an attractive site for mucoadhesives because of the ease of access, avoidance of first-pass metabolism and a relatively permeable and well-vascularised membrane. But the rapid removal of substances by mucociliary action becomes a big disadvantage for mucoadhesive administration. Bioadhesive microparticles are being investigated for local drug administration to the eyes (Genta et al, 1997). The disadvantage is the gel-like formulations can spread on corneal surface and blur vision. The GI tract is the most important route for drug administration. Mucoadhesives increase the retention of dosage form in the GI tract thereby increasing drug bioavailability. But the peristaltic waves within the GI tract and the constant turn-over of mucus make it difficult for non-specific bioadhesives to be retained long-term in the GI tract. Some specific bioadhesives and more sophisticated formulations, including micro- and nano- particles with polymeric coatings or highly specific 'biological ligands' like plant lectin were investigated for adhesion on GI tissue, which can specifically recognise and bind to N-acetyl glucosamine-containing complexes on cell surfaces and showed strong bioadhesion to intestinal tissue *in vitro* (Naisbett & Woodley, 1994).

1.5.3 Techniques used to evaluate adhesion of dosage formulations

This project focuses on the investigation of the adhesive profile of liquids coating the oesophagus, the techniques used to evaluate adhesion of formulations will mainly concentrate on the evaluation of bioadhesives within the oesophagus.

Tablets or capsules becoming lodged in the oesophagus is a relatively common problem. Solid dosage form adhesion to the oesophageal mucosa is not wanted because the adhesion of solid dosage form leads to dehydration of the mucosal surface and formation of a gel interface between the dosage and the epithelium. The drug diffuses from the solid dosage into the epithelium via the concentration gradient and corrodes the local tissue. Some techniques were developed to investigate the adhesion of the solid dosage forms to the oesophagus by measuring the force of detachment of the dosage from the surface of oesophageal mucosa and revealed that the force of detachment depends on the time left on the mucosa before removing the dosage form, the contact area and the natural variance of oesophagi (Marvola et al, 1982; Al-Dujaili et al, 1986; Honkanen et al, 2002).

On the contrary to solid dosage forms, long duration of adhesion to the oesophagus of a liquid formulation is desirable since it can form a layer on oesophageal mucosa to protect it against reflux and can act as a drug vehicle for local disease treatment.

An early technique was described by Ito et al (1990); bioadhesive polymers were screened using a glass tube coated with agar to mimic oesophageal tissue, the bioadhesive particles were mixed with blue dye and washed to mimic salivary flow. A colorimetric assay was used to determine the adhesion of particles. Dobrozi et al (1999) used everted rat oesophagus to measure the retention of liquid sucralfate formulations and showed that the sucralfate gel suspension was retained to a greater extent than non-gel formulations. This method has also recently been adapted for use by Richardson et al. (2004). A model described in detail by Batchelor et al (2002) that has also been used by Banning et al (1998), Young et al (1998) and Smart et al (2004) was the technique used in this project for measuring the retention of liquid sodium alginate

on oesophageal mucosa. The details of this apparatus and the experimental processes will be described in the next chapter.

1.6 ALGINATES

The first scientific studies on the extraction of alginates from brown seaweeds were made by a British chemist E.C. Standford at the end of the 19th century. He found some interesting properties from the extracted substance of brown seaweeds, including the ability to thicken solutions, to make gels, and to form films. However large-scale industrial production of alginates was not introduced until 50 years later. (Manufacturer's information; FMC Biopolymer)

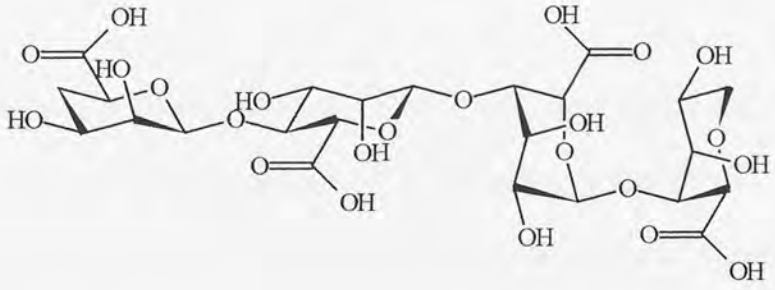
1.6.1 Origins of alginates

Alginates exist quite abundantly in nature. They occur in the cell walls and intercellular spaces of brown algae, such as *Phaeophyceae* and *laminaria hyperborea*. Their function is to supply the plant with strength and flexibility, which are necessary for plant growth in the sea. Some soil bacteria can also synthesize alginates as capsular polysaccharides (Smidsrød & Draget, 1996). Because of their ability to hold water, gelling, viscosity enhancing and stabilising properties, alginates are widely used industrially.

1.6.2 Properties of alginates

The first information about the sequential structure of alginates was given by Haug et al, (1967). They concluded that alginates are linear unbranched binary copolymers

containing β-(1, 4)-linked D-mannuronic acid (M unit) and α-(1, 4)-linked L-guluronic acid (G unit) residues. Their molecular structure is shown in Figure 1.3.



$$\mathbf{M\,(^4C_1)} \longrightarrow \mathbf{M\,(^4C_1)} \longrightarrow \mathbf{G\,(^1C_4)} \longrightarrow \mathbf{G\,(^1C_4)}$$

Figure 1.3 The structural units of alginate molecules

The chemical compositions of alginates are variable according to seaweed species and even within different parts of the same plant. They are not random copolymers, they consist of blocks of similarly or strictly alternating residues (i.e. MMMMMMM, GGGGGGG and GMGMGMGM), each of which have different conformational preferences and behaviour. The proportion and distribution of these blocks determine the chemical and physical properties. The block sequential analysis of alginate can be determined by ^{1}H and ^{13}C NMR spectroscopy (Grasdalen et al., 1979).

High M content alginates produce weaker, more-elastic gels, whereas high G content produce strong gels with good stability (except if present in low molecular weight molecules) (Manufacturer's information; Pronova Biopolymer). That may be explained by Smidsrød et al (1973) that the diaxial linkage in G-blocks largely hinder

the rotation around the glycosidic linkage, and may account for the stiff and extended nature of the alginate chain.

1.6.2.1 Viscosity

The viscosity of an alginate solution depends on the length of the alginate chain, the longer the chains the higher the viscosity at similar concentrations. Temperature influences the viscosity at a general rule; 1°C increase leads to a viscosity drop of approximately 2.5 % (Manufacturer's information; FMC Biopolymer). Rheologically, aqueous solutions of alginates have shear-thinning characteristics, termed pseudosplasticity, meaning that the viscosity decreases with increasing shear rate. In acid conditions alginate will precipitate or form gels. Mannuronic acid and guluronic acid have pK_a values of 3.38 and 3.65 respectively (Manufacturer's information; FMC Biopolymer). At pH values below the pK_a, the alginate is able to form a gel.

1.6.2.2 Ion-binding properties

The ion-binding properties of alginates are the basis for their gelling properties. The reactivity with calcium and the consequent gelling capacity is a direct function of the average length of the G-blocks. The alginates containing the highest GG-blocks possess the highest ability to form gels. The egg-box model explains this property (Grant et al., 1973). Free carboxylic acids (without counterion) have a water molecule H_3O^+ firmly hydrogen bound to carboxylate groups, Ca^{2+} ions can replace this hydrogen bonding, zipping guluronate, but not mannuronate, chains together in a supposedly egg-box like conformation (Fig.1.3). This binds the alginate molecules together by forming a junction zone. After gelation, water molecules are physically

entrapped by the alginate matrix, but are still free to move. The water-holding capacity of the gel is due to capillary forces.

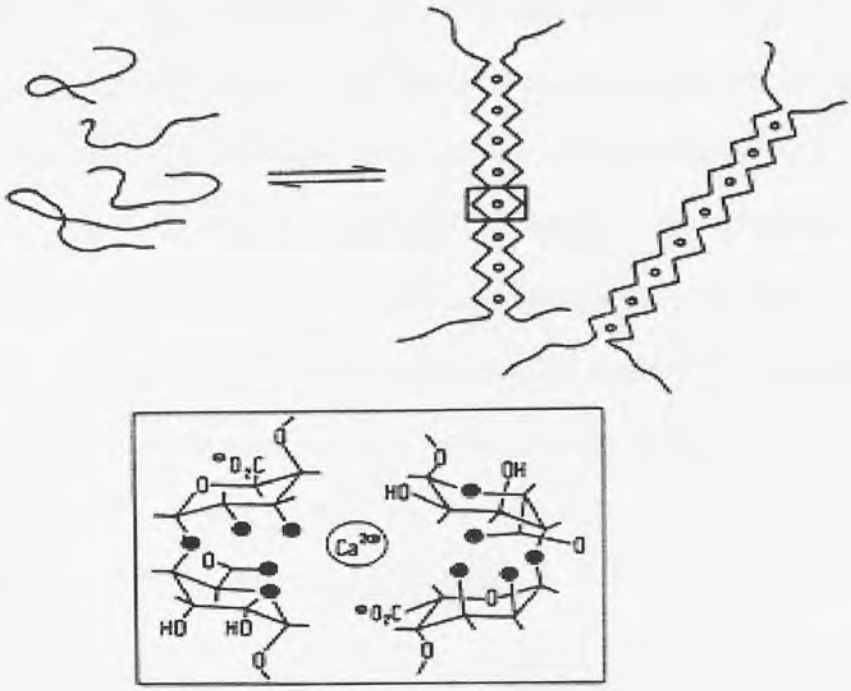


Figure 1.4 Schematic representation of the egg-box association of the poly-L-guluronate sequences of alginate crosslinked by calcium ions.

1.6.2.3 Stability of alginate

Being polysaccharides, alginates are susceptible to hydrolysis or degradation in strong acid and alkali, especially on heating. If stored in a dry, cool place without exposure to sunlight, dry powered, pure sodium alginate can be stored several months. In the deep freezer, sodium alginate may be kept for several years without significant reduction of molecular mass (Smidsrød & Draget, 1996).

1.6.3 Functions of alginates

Alginates and alginic acids have been used in a wide variety of applications for more than 50 years. The most commonly used alginates are sodium alginate. Other important compounds are potassium, ammonium, magnesium, and calcium as well as alginic acid itself and propylene glycol alginates (PGA). Water-soluble alginates can act as stabilizers and thickening agents in systems consisting of particles or droplets dispersed in water, like in ice cream and salad dressings. If a thin layer of alginate gel or solution is dried, a film a coating is formed. This property is used in paper making applications. In textile printing applications alginate is used to control the flow characteristics of printing pastes and help to fix the colours onto the fabrics. (Manufacturer's information; FMC Biopolymer)

JECFA (Joint FAO/WHO Expert Committee on Food Additives) has granted alginate an Acceptable Daily Intake (ADI) of "not specified". This is the highest possible for food additives (Manufacturer's information; FMC Biopolymer). Animal toxicity experiments show that sodium alginate is well tolerated at doses up to 500 mg/kg. So alginates are widely used in pharmaceutical applications. Gaviscon® products introduced more than 30 years ago contain alginate for treating the patients who are suffering from GORD. The alginate component creates a 'raft' floating on the gastric contents to reduce their reflux into the oesophageal lumen thus protecting the oesophageal mucosa from the acid-pepsin gastric reflux attack. The strong adhesive property of alginate was used to make alginate adhesive tablets (Hunt et al., 1987) that showed the same adhesive ability as typical commercial oral mucosa-adhesive tablets (Teijin Ltd., 1980; Miyazaki et al., 1995).

1.7 AIMS AND OBJECTIVES

Aims: To investigate the retention profile of liquid alginate on oesophageal tissue and its capacity in protection of the oesophageal epithelium from the damage caused by gastric refluxate.

Objectives:

- To study the ability of aqueous alginate to adhere to the oesophageal tissue and determine the factors within the gastric refluxate and the pH of the washing material (to mimic saliva) that control the adhesion of an alginate solution to oesophageal tissue

- To investigate the factors that influence the viscosity of an alginate solution and the effect of viscosity of alginate on retention. The effect of pH, concentration, presence of mucin and/or pepsin and the duration of stirring on the viscosity of an alginate solution was measured.

- To develop a cell culture technique to measure both adhesion of alginate and the protection it offers to individual cells against damage caused by components of gastric reflux.

- To develop a technique using a Franz-cell to measure the diffusion rate of acid and pepsin through an adhesive alginate solution that may be used to measure the protective capabilities of alginate solution on oesophageal tissue

- To measure the protection offered by an adhesive solution of alginate to the oesophageal tissue, a new technique was developed using microscopy and a score system of epithelial injury to assess the epithelium damage caused by components of gastric reflux (acid and pepsin).

CHAPTER 2 RETENTION STUDY

2.1 INTRODUCTION

This study investigated the adhesion of aqueous sodium alginate solutions to oesophageal tissue. An adhesive layer resident on oesophageal tissue can act as both a means of enhancing oesophageal defence against gastric reflux and a promising drug delivery tool. Different factors that may affect retention were studied. Analysis was via fluorescein dye associated with alginates, fluorescent covalently labelled alginates, and radio-label associated with alginates. Fluorescently labelled beads were introduced to mimic drug particles within the alginate to study the possibility and capability of aqueous alginate as a drug delivery system. Furthermore microscopic images were collected to confirm that the liquid alginate was retained on the oesophageal surface.

2.2 MATERIALS AND APPARATUS

2.2.1 Materials

2.2.1.1 Preparation of porcine oesophageal tissue

Porcine oesophagus was collected from the abattoir on the day of sacrifice and transported to laboratory. In the laboratory, the outer muscular layers of the oesophagus were removed by cutting through the muscle layer to expose the white

inner epithelial tube, and then the muscular layer was carefully peeled off, taking care not to damage the inner mucosa during this process. The epithelial tube was washed gently with tap water to clear the food residue within the oesophageal tube, and then stored at -70 °C until required. Prior to use, the frozen oesophageal tube was thawed at room temperature within a sealed plastic container or via immersion in saline solution.

2.2.1.2 Hydrated dialysis membrane and Parafilm®

Hydrated dialysis membrane was used as an alternative biological substrate to porcine oesophageal tissue. When wet, the surface of a dialysis membrane is moist and smooth, similar to oesophageal tissue; the moistness may contribute to increase the surface adhesion properties. Parafilm® was used as an alternative biological substrate to porcine oesophageal tissue as a negative control. The function of Parafilm® is to create a seal and it is impermeable to water thus retention on such a substrate should be minimal.

2.2.1.3 Sodium alginates

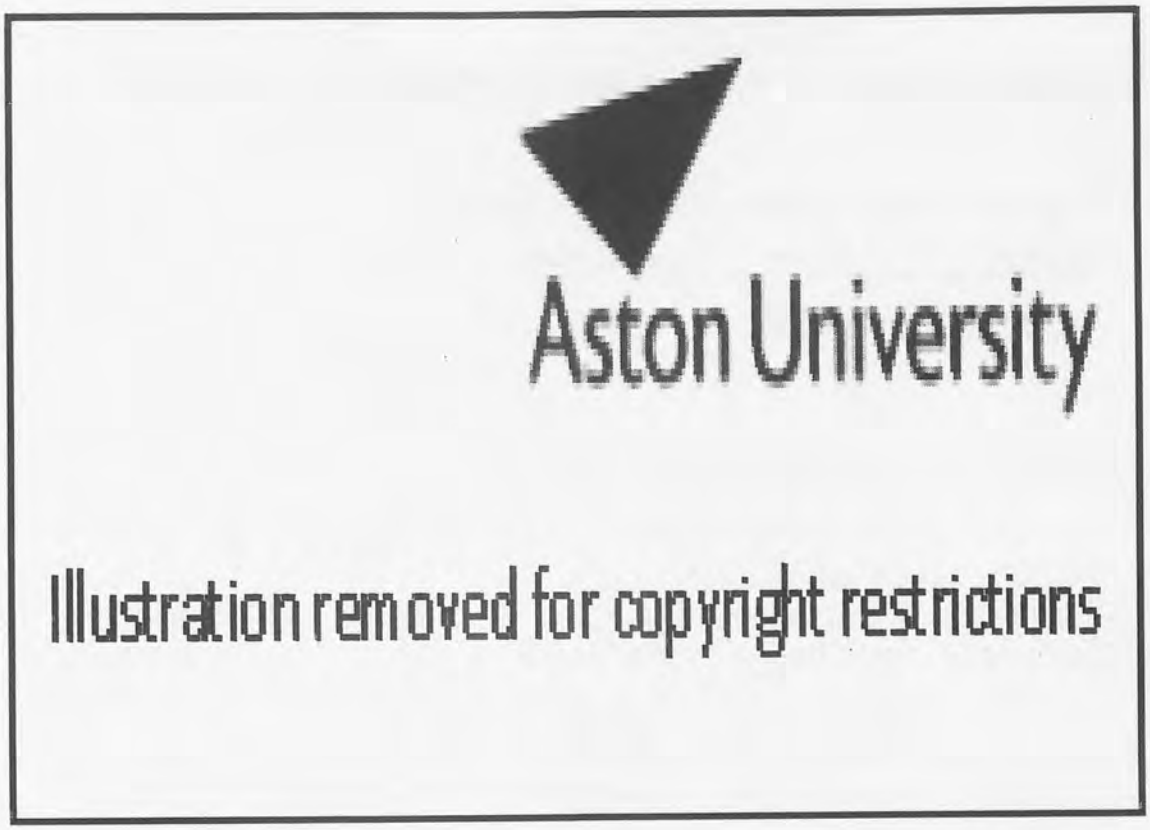
Sodium alginate powders were kindly supplied by FMC Biopolymer (Norway) and stored at a temperature of 4 – 7 °C, protected from sunlight. Under these conditions sodium alginate is reported to be stable for several months (Smidsrød & Draget, 1996). The chemical and physical properties of alginates used in this study are listed in Table 2.1.

A 2 % w/v concentration was chosen in most experiments except when concentration was the investigated factor. The dry weight of sodium alginate was used for calculating the required mass to produce the set concentrations in each experiment. The alginate solutions were prepared using a Heidolph rotary mixer with a 20 mm diameter, four-blade propeller. Double distilled water was stirred at a speed of approximately 1800 rpm and a set mass of sodium alginate powder was added slowly into the vortex caused by the propeller. Then the rotary speed was increased to 2000 rpm and left to mix until all the alginate powder was dissolved and produced a homogenous solution.

Three methods were used to label alginate solutions in the retention study. They were fluorescent non-covalently labelled by preparation in a solution of disodium fluorescein; fluorescent covalently labelled by covalent attachment of a fluorescent molecule; and non-covalently radio-labelled by mixing a radioactive chemical Technetium ($^{99}Tc_m$) with an alginate solution. In the drug delivery study, several fluorescently-modified beads with positive and negative surface charges that mimic drug particles were used dispersed within the alginate solutions. For the microscopy

investigations fluorescent non-covalently labelled alginate solutions and fluorescent beads mixed in alginate solutions were prepared. The details of the labelling procedures are described in detail in the following sections.

2.2.1.4 Simulated saliva and materials used in the retention model



The tissue was washed with a solution to mimic saliva flow within the retention study. Due to the large volumes of washing medium it was not practical to use real saliva thus, double distilled water was used in the place of saliva as a washing medium. Pepsin is an important component of gastric reflux that may affect the alginate retention, so pepsin solutions (Sigma pepsin A, P7012, sourced from porcine gastric mucosa MW 35 KDa, 2991 units/mg in solid) 0.1 % w/v was used as washing medium in the retention study to compare to distilled water. Acidic Gastric reflux was

investigated in this study, thus buffer solutions with a range of pH values were prepared to investigate their effect on retention. Preparation of the buffers is outlined in table 2.2. Difference types of buffer were used to cover the pH range from 2-8.

2.2.1.5 Radiochemical – Technetium

Alginate solutions were labelled with a radioactive label to eliminate the effects of acid pH on the fluorescence observed and to quantify the retention of opaque solutions. An additional benefit of radio-label is that the percentage retained on the tissue surface as well as that removed was quantified. Element 43, Technetium, in the periodic table has twenty-two isotopes with masses ranging from 90 to 111 that are all radioactive. Technetium is produced as a fission product from the fission of uranium in nuclear reactors and has several long-life radioactive isotopes, ^{97}Tc (Half-life $t_{1/2}$) = $2.6x10^6$ days), ^{98}Tc ($t_{1/2}$=4.2×10^6 days), ^{99}Tc ($t_{1/2}$ = $2.1x10^5$ days), $^{95}Tc_m$ ($t_{1/2}$=61days). In this study $^{99}Tc_m$ was used because of its short half-life (T 1/2 = 6.01 hours); this short half-life and the gamma energy emitted mean that technetium has been used in previous studies that measure oesophageal adhesion *in vivo* (Potts et al. 1997).

2.2.2 Apparatus

2.2.2.1 Retention models I and II

There were two types of models used in this study for the experimental process. Both had the same mechanism of operation but were different in design. The main structures of both of the models had three parts, a mounting platform whose incline angle could be adjusted, that supported a piece of oesophageal tissue; a peristaltic

pump provided an appropriate washing medium that flowed over the tissue surface; and a collector vial under the platform to collect the eluate for quantification of retention. A schematic representation of the model is shown in Figure 2.1

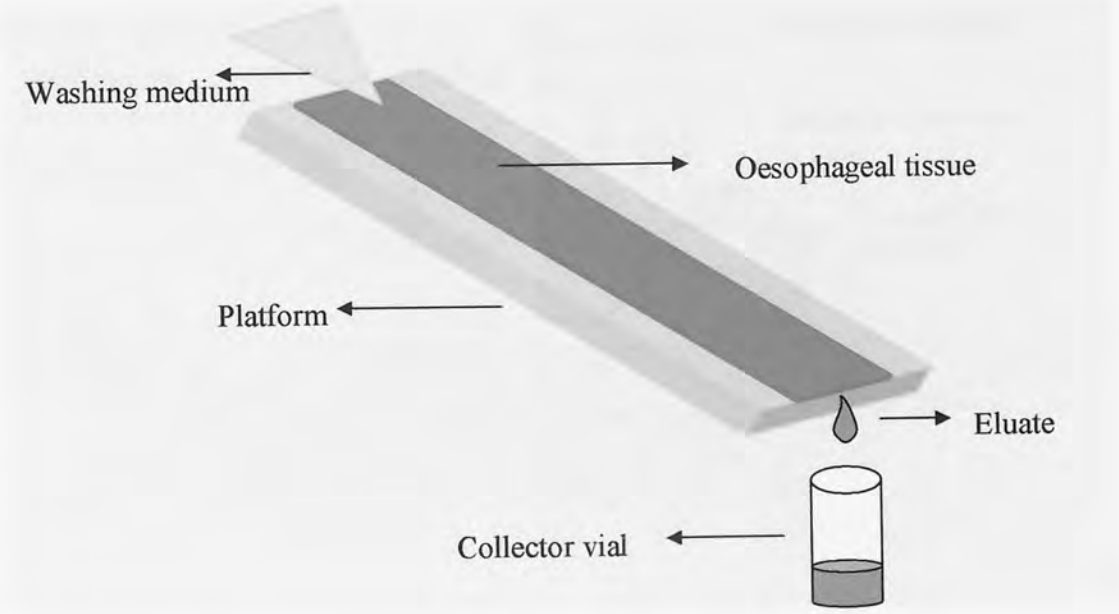


Figure 2.1 Schematic representation of basic retention model apparatus

Retention model I

This model apparatus was composed of several instruments shown in Figure 2.2. On the top a cabinet holds the mounting platform to support the tissue substrate. A thermometer was placed in the front wall of the cabinet, on the bottom a water bath supplied the humidity and temperature that could be adjusted during the whole experimental procedure. According to the reading of the thermometer, the water bath (TE-7 Tempette®) was adjusted to control the temperature in the top cabinet where the experiment was performed to mimic the conditions *in vivo*, the temperature within the oesophageal cavity is 37 °C and the humidity is assumed to be close to100 % RH. On

both sides of the top cabinet two sealed glove access points allowed experimental operation without altering the environmental conditions.

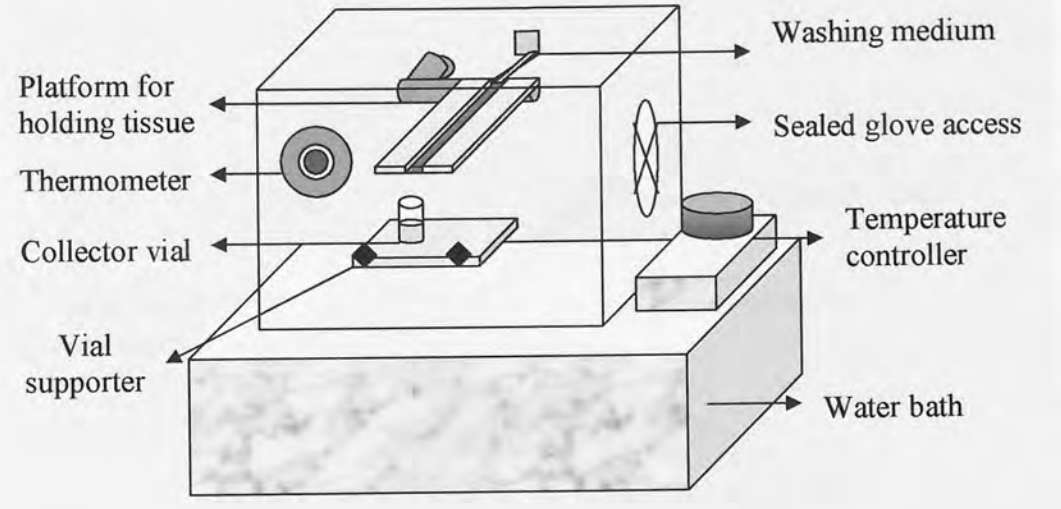


Figure 2.2 Schematic representation of retention model I

A peristaltic pump (Watson Marlow model 202) accurately controlled the flow rate of the washing medium. In order to evenly distribute the washing material over the surface of the oesophageal tissue, the tube in conjunction with the pump was split into four separate tubes. 7 mL vials under the mounting platform collected the eluate at set time points for subsequent analysis.

Retention model II

Based on the model I, model II was smaller and easier to operate and move from place to place (this was particularly useful for radiochemical labels). The shape and structure is closer to the oesophageal tube physiologically. This model was used in most

retention experiments, particularly the fluorescently labelled aqueous alginate (non-covalent label), radio-labelled alginate retention study and aqueous alginate as drug delivery tool research as well. The whole apparatus was made of Perspex®. Figure 2.3 schematically shows the structure of this apparatus.

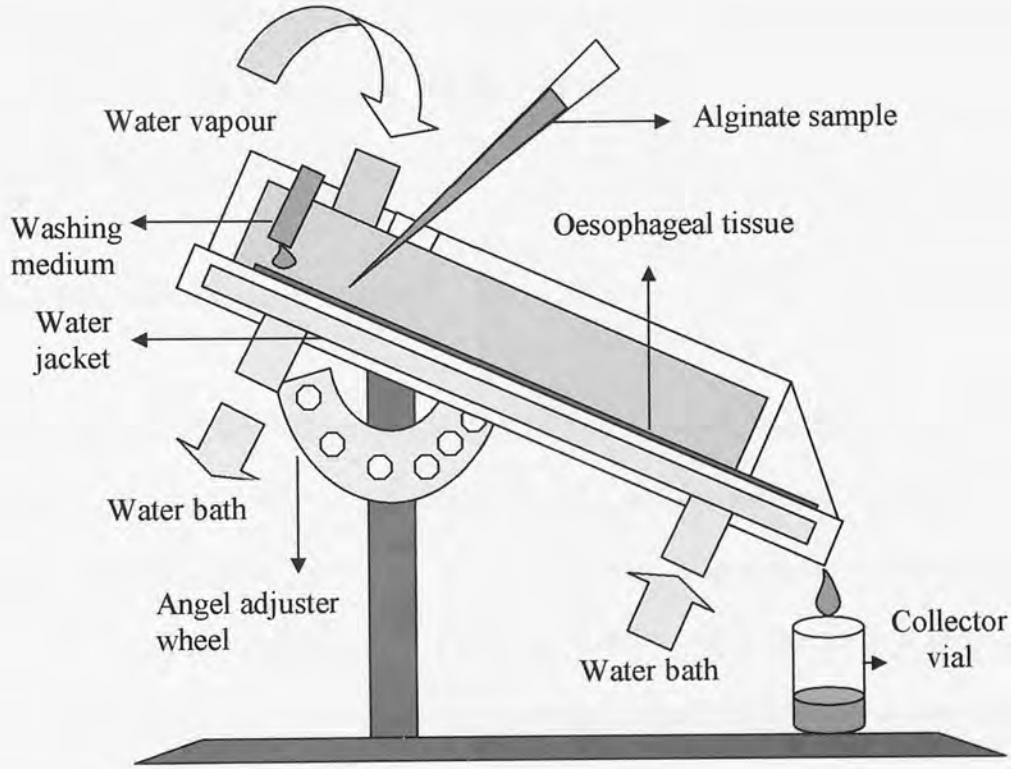


Figure 2.3 Schematic representation of retention model II

The oesophagus is enclosed within a Perspex® tube that has a tightly fitting cover that is secured once the tissue is in place. There are two holes on this cover; one is for dispersing 37 °C washing medium through a plastic tube that was connected with a peristaltic pump (Nachem miniplus 3). The flow rate was accurately controlled by this pump to a rate of 1 mL/minute. Another hole was used to input water vapour to maintain the whole tube close to the physiological humidity of >80 % RH.

The water jacket under the platform maintained the temperature of the oesophageal tissue mounted on the bottom of the tube at around 37 °C. The water jacket was linked to a temperature controlled water pump (Churchill). The water flowed from the lower inlet to the higher outlet. Under the platform an angel adjuster was used to set six angles for the platform, 0°, 15°, 30°, 45°, 60°, 75°. There are six holes on the angle adjuster wheel, a hole on the support column. An angle was set through a bolt that fixed one of the angle wheel holes with the column hole. A spirit level was used to adjust the apparatus horizontally.

2.2.2.2 Dissolution apparatus type I

Dissolution is defined as the process by which a known amount of solid drug substance goes into solution per unit of time under standardised conditions. The primary goal of dissolution testing is to be used as a qualitative tool to provide measurements of the bioavailability of a drug as well as to demonstrate bioequivalence from batch-to-batch. In this study USP dissolution apparatus (type I) was used with a modified experimental procedure.

Figure 2.4 schematically shows the structure of the apparatus I. The vessel is dome-shaped, 160 - 210 mm high, internal diameter (upper) 98 – 106 mm, nominal capacity is 1000 mL. The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel and rotates smoothly and without significant wobble. The distance between the inside bottom of the vessel and the basket was maintained at 25 $\pm$ 2 mm during the test.

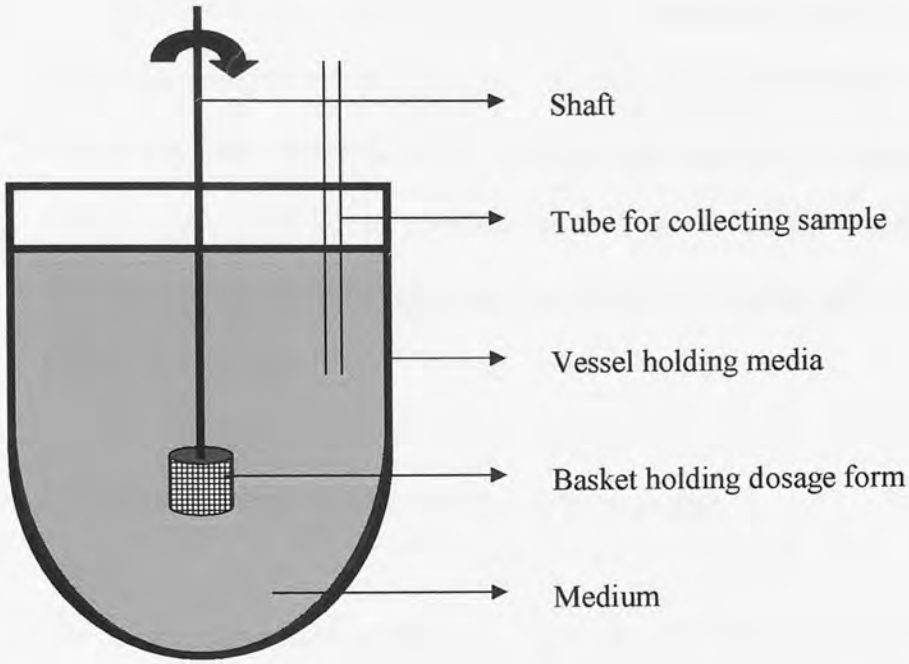


Figure 2.4 Schematic representations of USP dissolution apparatus

The method used in this study was slightly changed from the general operation and will be described in detail in method section (2.3.3).

2.2.2.3 Fluorescence spectrophotometer

The collected fractions from the two retention models and the dissolution apparatus I were analysed by fluorescence spectrophotometer. A Spectra MAX® Gemini XS plate reading spectrophotometer was used.

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 is 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 provided a clear advantage of fluorescence spectroscopy as a means of analysis. A typical spectrophotometer contains an excitation source, a sample cell and a fluorescence detector (Manufacturer's handbook of fluorescence spectrometer Spectra MAX® Gemini XS).

2.2.2.4 The gamma counter and theory of operation

The radio counter used in this study was Packard Cobra Model 5005 gamma counter. The first requirement of a gamma counting system is the detection of gamma radiation. The majority of gamma ray detectors employ the combination of a scintillation crystal and a photomultiplier tube (PMT).

Gamma rays traversing a sodium iodide (NaI) crystal interact with electrons in the crystal to produce scintillations of light. The scintillation process can be viewed in three steps. First, the gamma ray interacts with the one or more electrons in the crystal giving up all or part of its energy. Second, the energetic electron(s) in turn interact with other electrons raising a number of them to excited electronic states in the crystal. Finally the excited electrons return to their ground states by various mechanisms, some of which produce light by generating photons. The final scintillation or light flash contains a total number of photons proportional to the energy lost by the gamma ray in the first step. So the important feature of the scintillation process is that the amount of light produced by each gamma ray is proportional to the amount of energy lost by the gamma ray in the crystal. Thus, a scintillation detector not only detects gamma ray, but sorts and counts them according to their energies.

2.2.2.5 Microscopy

In biology, microscopic techniques are regularly used as a means of viewing and analysing the histological structure and formation of biological materials. In this project light and fluorescence microscopes were used to view an alginate layer present on the oesophageal tissue surface and measure the depth of the layer. The principals of the two microscopes used are described below.

2.2.2.5.1 Light microscope

Microscopy is used to provide magnification of a specimen. A compound microscope enlarges the observation of a given sample. A compound microscope has two convex lenses for generating an image. One lens, termed the objective, has a short focal length, 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. An illumination system is very important for a compound microscope. The illumination system of the standard optical microscope is designated to transmit light through the translucent object for viewing. In a modern microscope, it consists of a light source, usually an electric lamp and a convex lens for condensing the light to provide a bright and uniform illumination in the region of object under observation. To produce the optimum image, it is necessary to adjust the lens so that the entire image in the observation region is brightly and evenly illuminated. The aperture of the objective lens can be adjusted to collect the light as required.

2.2.2.5.2 Fluorescence microscope

In order to visualise the fluorescently labelled alginate layer adhere to the oesophageal tissue surface, a light microscope was used in fluorescent mode. The principal of fluorescence is explained in 2.2.2.3. A fluorescence microscope works in a similar way. 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 a chromatic beam splitter or a 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 book by Tanke & Herman (1998) provides an excellent review on the principals and uses of fluorescence microscopy. A Zeiss Axioskop microscope was used to take pictures in this study.

2.3 METHODS

In this project several different labelling methods were used, including fluorescent non-covalent label, fluorescent covalent label, fluorescent beads as a label mixed with alginate and radio-(non-covalent)-label. The retention procedures in each case were similar. The radiolabel retention procedure will be described separately (Section 2.3.2.3) as it required additional safety precautions.

2.3.1 Alginate Label Methods (fluorescent dye or beads)

2.3.1.1 Preparation of fluorescent non-covalently labelled alginate solution

Disodium fluorescein 0.125 g was dissolved in 100 mL distilled water from which 1mL solution was removed and taken out to dissolve in 100 mL distilled water. Sodium alginate powder was prepared within the second solution to make 2 % w/v liquid. The mass of powder was calculated from the dry weight of the alginate that the manufacturer supplied. Such as alginate H120L dry weight is 86.9 % w/v. if 10 mL 2% w/v solution need to be prepared, the mass of the powder was produced by 2 % x 10/ 86.9 % = 0.2301 g.

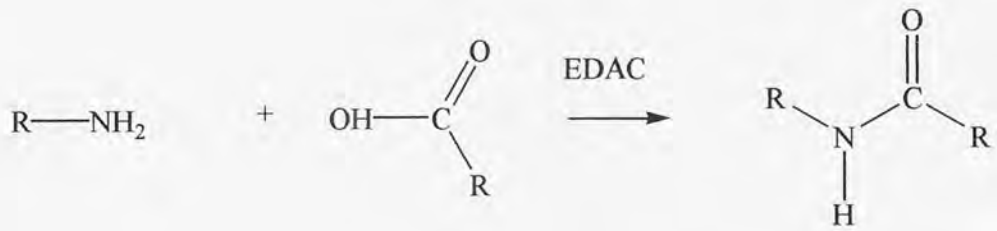
In this case, the water-soluble fluorescent dye molecule does not covalently bind to the alginate molecule, but it is easily prepared and batch-to-batch variation was very small.

2.3.1.2 Preparation of fluorescent covalently labelled alginate

Approximately 35.43 mg Fluoresceinamine was dissolved in 1 mL DMF (Dimethyl Formamide) and the solution kept at 4°C until used. 1 g sodium alginate was dissolved in 100 mL distilled water and stirred until fully dissolved. The pH was adjusted to 4.75 using 0.1 M HCL and 0.01 M NaOH. 100 mg EDAC, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide and 200µl fluoresceinamine solution were added. This solution was stirred at 4°C for 30 minutes after which the pH was adjusted to 4.75 again and a further 100 mg EDAC was added. The solution was stirred at 4°C for 30 minutes and a final adjustment to pH 4.75 was made and the solution was stirred overnight at 4°C. The covalently bonded fluoresceinamine-alginate solution was

adjusted to the original pH 6 using 0.1 M NaOH and 0.01 M HCl and dialysed against distilled water until the dialysate showed no absorbance at 490 nm (the absorption wavelength for fluoresceinamine) against a blank of double distilled water. The alginate solution was freeze-dried and the resulting labelled powder was stored at 4°C.

In Figure 2.5 the reaction couples an amine to a carboxylic acid using a carbodiimide which is in EDAC. EDAC itself hydrolyses in aqueous solution (that is why two aliquots were added half an hour apart). The EDAC was present in great excess. The amount of fluoresceinamine stated in the method is equivalent to a 1:250 fluoresceinamine: carboxyl ratio, however the labelling was less than this. The free fluoresceinamine molecules were extracted in the dialysis stage.



Fluoreceinamine Alginate Labelled alginate

Figure 2.5 Schematic of reaction between fluoresceinamine and alginate

The free dye molecules were removed from the labelled solutions after the reaction had finished via dialysis. There are two methods to assess whether the free dye was removed completely. One is to determine the fluorescence intensity of the dialysate. If the intensity shows no absorbance against a blank, it means the free dye molecules have been cleared. Another method was gel filtration chromatography; a column that contains a stationary phase of porous beads with a well-defined range of pore sizes.

Molecules that are smaller than the pore of the beads fit inside the pores and become the internal mobile phase and the external mobile phase was the gel filtration column. Molecules that are large with respect to pore size are eluted first. As the molecular weight of the dye is much lower than the labelled alginate molecules, the two entities should be separated and will be seen as two peaks in the gel filtration chromatography.

Before dialysis of the labelled alginates, 1mL labelled alginate solution (10 mg/mL) was run down the column and two bands of colour moving through the filtration column were observed. Theoretically the first band was the labelled alginate. The second band was the free dye. The result in Figure 2.6 shows the two peaks of fluorescence intensity.

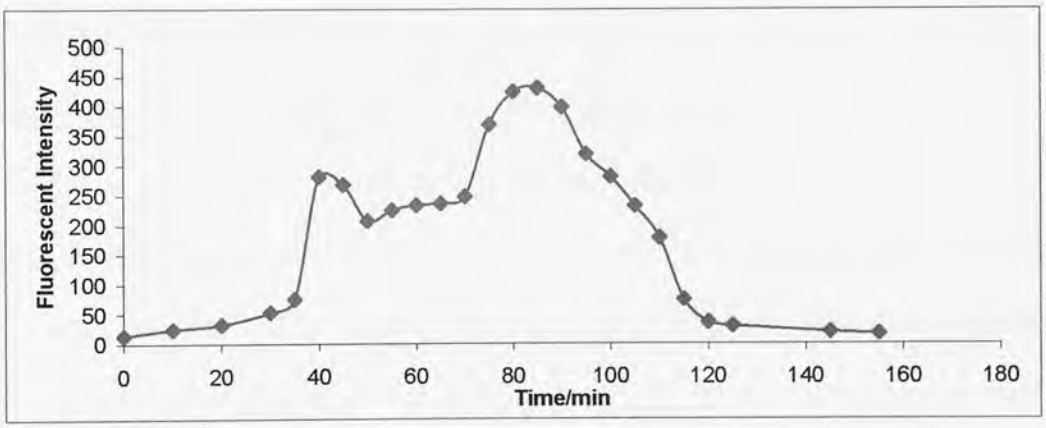


Figure 2.6 Fluorescence intensity of covalently labelled alginate (LF120) before dialysis against distilled water using filtration chromatography

After dialysis of the labelled alginate against distilled water for two weeks, only one peak was observed (Figure 2.7) indicating that very little unbound dye was present in the solution.

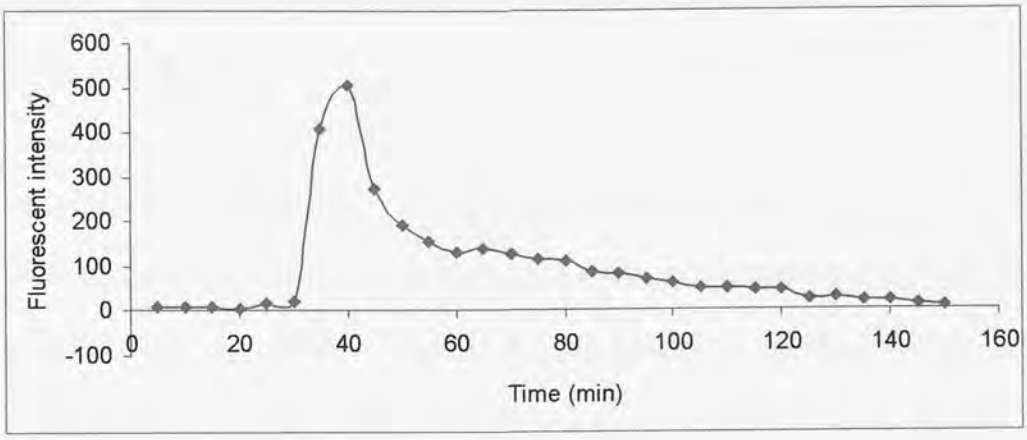


Figure 2.7 Fluorescence intensity of covalently labelled alginate (LF120) after dialysis against distilled water using filtration chromatography

Unfortunately although the covalent bond was formed between free dye and alginate molecules, the batch-to-batch variation was very large. The variation of fluorescence intensity of the batch-to-batch labelled alginates is shown in Table 2.3. Viscosity is a very important factor that can significantly affect alginate retention, the covalent labelling process led to changes in alginate viscosity (Table 2.3). That indicates that the chemical structure of the polymer molecule chain had changed, thus the retention of this alginate may not be typical of the true result. Because of these shortcomings the covalently labelled alginate by fluoresceinamine molecule was not used in the study.

Table 2.3 The fluorescence intensity of covalently labelled LF120 solution (2 % w/v) using a spectrophotometer at λ= 515 nm

Batches Number	Fluorescence Intensity	Viscosity mPa.s at shear rate 10 s^{-1})
1	6791.77	415
2	12110.2	460
3	1577.02	532
Non-label	N/A	1416

2.3.1.3 Preparation of an alginate solution containing fluorescently modified beads as a marker

Fluorescently-modified beads were introduced into the study not only to investigate the alginate retention, but also to test the possibility and capability of aqueous alginate as a drug delivery system targeted to the oesophagus. Three fluorescently-modified beads, sulfate ($-SO_4^{2-}$), carboxylic ($-COO^-$), amine ($-NH_4^+$) (Sigma) with different surface charges, were used to mimic drug particles. The diameter of the beads was approximately 2 μm. Sulfate beads have bivalent negative charges, carboxylic beads have negative charge, and amine beads have positive charge. 1mL beads suspension was diluted into 50 mL double distilled water. To investigate whether the alginate solution would increase the retention time, each bead suspension was made up to 2 % w/v alginate solution to compare with the aqueous dispersion of the beads.

2.3.2 Retention procedure (model I/II)

For the fluorescent non-covalently labelled alginate and fluorescently-modified beads within the alginate (no data for the retention of fluorescent covalently labelled alginate), the retention procedures were the same and are described in detail below.

The porcine oesophageal tissue, prepared as described in 2.2.1.1, was removed from the freezer and allowed to thaw in an isotonic solution of sodium chloride (0.9 % w/v) at room temperature. The tissue was cut to size and mounted on the platform within the chamber shown in Figure 2.1, Figure 2.2 and Figure 2.3. Prior to dispersing a fixed dose (1 mL) of labelled alginate solution onto the tissue surface, the angle of the mounting platform was adjusted to a set angle, generally it was 30 degrees to the

vertical. The washing medium flow rate was set up accurately at 1mL/min. It flowed evenly over the tissue and alginate for up to 30 minutes. Eluted material from the tissue surface was collected in vials at fixed time points. The chamber temperature was controlled at physiological temperature between 36.5 and 37.4 °C with humidity close to 80-100 % RH. Each experiment was repeated five times to calculate the average value. For the fluorescently labelled alginate, measurement of the eluate using a microplate spectrofluorometer (Spectra MAX® Gemini XS) enabled calculation of the amount of the applied dose retained at any time point via a calibration curve.

2.3.2.1 Calibration line

Calibration lines were prepared for both of the fluorescent non-covalently alginate solution and fluorescent-modified beads labelled alginate solution. A 2 % w/v stock solution of alginate containing the level of the dye described previously was prepared, from which a series of concentrations of liquid alginate were made; 0.5 %, 0.25 %, 0.1 %, 0.05 %, 0.01 %, 0.005 %, 0.001 %, 0.0005 %, 0 % (w/v). The g/mL was used as the unit of the concentration of the calibration solutions. For each experiment fresh calibration curves were prepared as differences may occur because the experimental operation and the nature of dose may vary between experiments.

Because of the different emission intensity, two examples of calibration curve were produced, one for fluorescent non-covalently labelled alginate shown in Figure 2.8, one for fluorescent-modified beads labelled alginate solution in Figure 2.9. Each curve was produced from the average of 4 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.

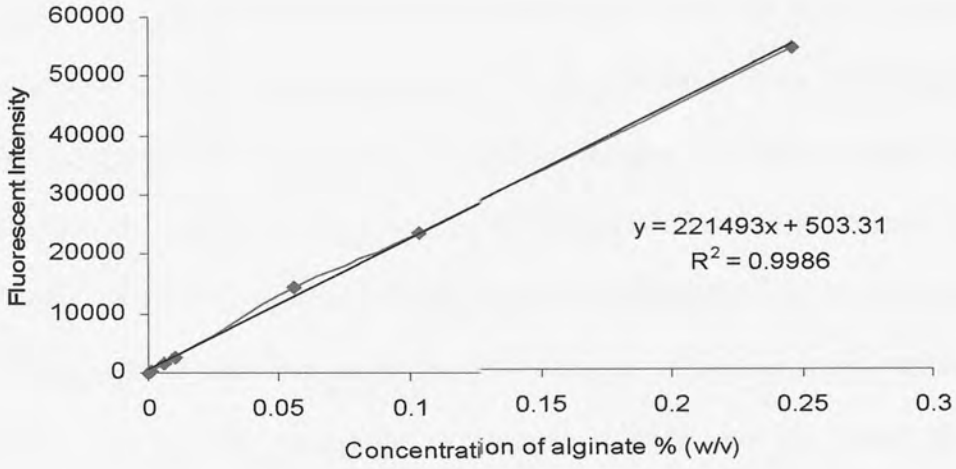


Figure 2.8 Calibration curve for fluorescent non-covalently labelled alginate solution

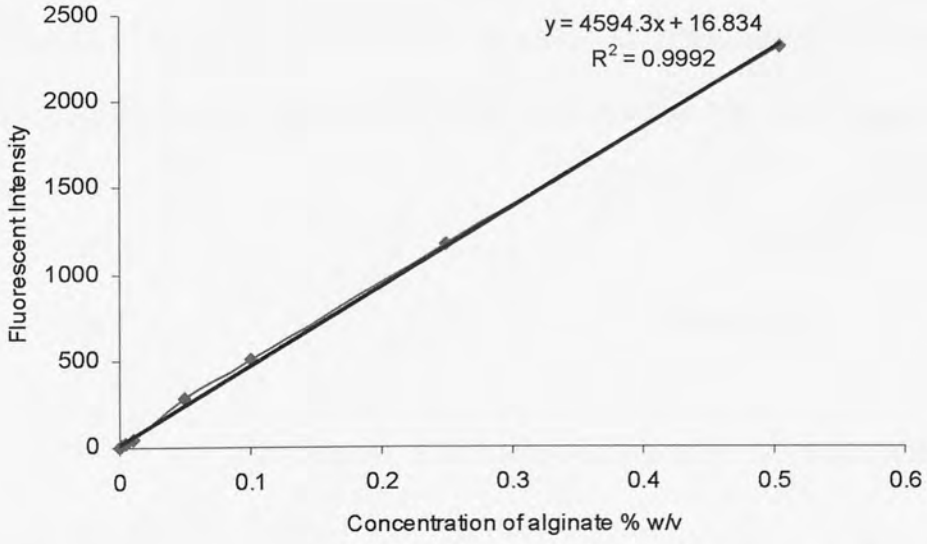


Figure 2.9 Calibration curve for fluorescently-modified beads labelled alginate solution

2.3.2.2 Calculation of retention

The calibration curve describes the relationship between the fluorescence intensity and the concentration of alginate solution. 0.2 mL of each eluate at each time point was taken to measure the fluorescence intensity so that the results were directly comparable to the concentration values from the calibration curve. The volume of each eluate was recorded. The mass of applied alginate solution in each eluate was calculated by multiplying the concentration with the volume of this eluate. Adding up the mass of each eluate washed off the tissue together and divided by the total alginate solution applied was used to get the percentage of alginate solution washed off the tissue. The retained percentage of alginate solution was calculated by 100 % subtracting the washed off percentage. The calculations in this process are detailed below.

$$\Delta M = M_1 - M_2 \qquad \text{Equation 2.1}$$

ΔM is the mass of alginate solution applied on tissue; M_1 is the weight of syringe prior to dispersing the alginate solution, M_2 is the weight of syringe after dispersing the solution.

$$Y = mX + c \qquad \text{Equation 2.2}$$

This is the calibration curve equation. Y is fluorescent intensity; X is the concentration of the alginate solution. m and c are two constants.

For each eluate, the fluorescence intensity Y was measured by the spectrophotometer, so that the concentration of alginate solution in each eluate was calculated by equation 2.3.

$$X = (Y - c)/m \qquad \text{Equation 2.3}$$

The mass of the alginate solution in each eluate M_t (t means time points) was calculated by concentration of alginate solution X multiply with the volume V (Equation 2.4)

$$M_t = X * V \qquad \text{Equation 2.4}$$

So the percentage of alginate solution A_t % washed off tissue at each eluate was expressed as equation 2.5.

$$A_t\ \% = Mt/\Delta M * 100\ \% \qquad \text{Equation 2.5}$$

Then the total percentage of alginate solution washed off tissue at some time point was expressed as equation 2.6

$$A_N\ \% = (A_1 + A_2 + A_3 + \ldots + A_N) * 100\ \% \qquad \text{Equation 2.6}$$

All the retention procedures including fluorescent non-covalently alginate solution and fluorescently-modified beads labelled alginate solution were calculated according to the same method. The factors investigated to affect the retention of the alginate solution were listed in 2.3.4.

2.3.2.3 Preparation of Radiochemical ($^{99}Tc_m$) non-covalently labelled alginate solution and experimental procedure

Because of the radioactivity of the element Technetium that was used in the retention studies, the experimental procedure is different from the fluorescent labelled one.

A strip of *in vitro* porcine oesophageal tissue was mounted onto the platform (model II described in detail in 2.2.2.1.2) that was maintained at 37 °C. A dose of labelled liquid material was dispensed onto the surface of this substrate. Washing media (usually double distilled water) was run over the surface of the substrate and the eluate was collected at designated time points for analysis. A special procedure was introduced as followed.

A preparation of about 10-20 MBq of technetium tin colloid within 5 mL was supplied by City Hospital, Birmingham. This material was kept within a lead pot for the duration of the experiment and was only opened for short periods to allow access to the material.

0.1 mL of this solution contained 0.2-0.4 MBq of radioactivity. 0.1 mL of the technetium solution was mixed with 1.5 mL solution of alginate. The syringe used to dispense the radiolabel was placed into a solution of 5 % Decon. The radiolabel was blended with the polymer solution in a screw top vial using a vortex mixer for 30 seconds until homogenous.

Approximately 1 mL of the blended mix was drawn into a clean syringe; this syringe was then weighed using a weighing boat on a 4 decimal place balance. The volume

(approx 1 mL) was then dispensed onto the tissue surface and the syringe was reweighed to calculate the exact mass that has been added (the density was assumed to be 1 g/mL). When handling the syringe it was placed within a test-tube to avoid contamination of surfaces with radiolabel.

A further volume (0.5 mL) of the original blend was drawn into a syringe and the syringe was weighed. This volume was then dispensed into a scintillation tube and the syringe was reweighed. This scintillation tube acted as a control to measure the counts per unit mass of our blended formulation, it was measured as the same time as all other samples.

Once the volume of material had been dispensed onto the tissue surface the eluate was collected directly into scintillation tubes. Once material had been collected the scintillation tubes were capped to prevent spillage. All apparatus was placed on a tray that could collect radiochemical material should there be a spillage.

2.3.2.3.1 General safety for radiochemical procedure

Because of the radioactivity of technetium, special rules needed to be obeyed; all workers were monitored via both a body badge worn at chest height and a finger guard worn on the middle finger of their lead hand. Workers were encouraged to stand back from their apparatus at times where they were not taking a sample. All used plastics were placed into a Decon 90 solution (5 % v/v) at the far side of the bench. All contaminated plastics were locked in the fridge for a minimum of 48 hours to allow natural decomposition prior to disposal via the designated sink and bin within the laboratory.

2.3.2.3.2 Calculation of retention

The mass of alginate solution washed off the tissue was calculated by the following two equations.

$$Mc / Rc = Mx / Rx \qquad \text{Equation 2.7}$$

$$Mx = Mc * Rx / Rc \qquad \text{Equation 2.8}$$

Mc: mass of the 0.5 mL control solution;

Rc: radio activity of the 0.5 mL control solution counted by the gamma counter;

Rx: radio activity of alginate solution in each eluate that was counted by the gamma counter;

Mx: mass of the alginate solution in each eluate at the designed time points which was calculated from the other three known values.

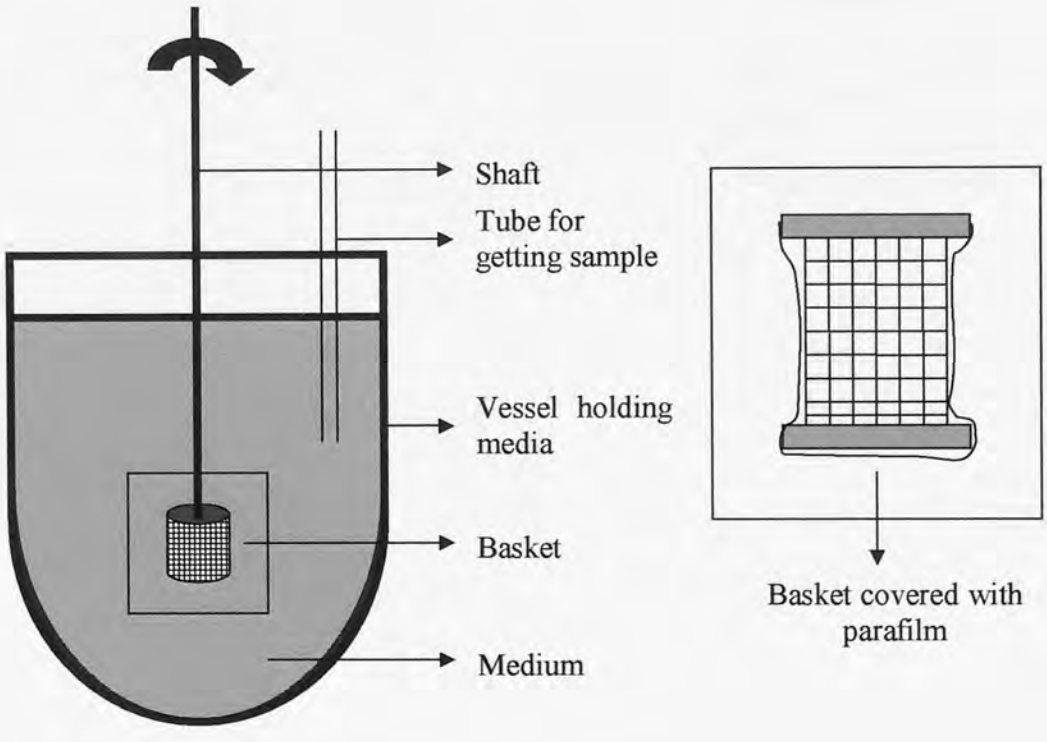
Then the total percentage of the alginate solution washed off the tissue was produced from equations 2.5 and 2.6.

2.3.3 Retention using dissolution apparatus I

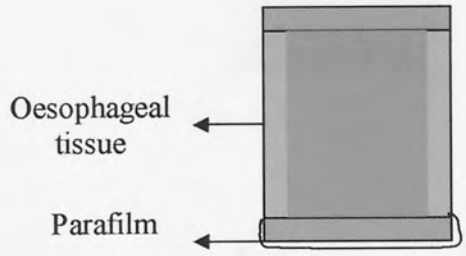
The dissolution apparatus I (Hanson Research) was used in this study. Six vessels holding 750 mL distilled water were fixed in the water bath in which the temperature was controlled at 37 °C. The rotary speed, raising and lowering of the six shafts, was controlled by a computer system. Prior to pushing the oesophageal tube on to the

baskets at the end of the shafts, the basket was covered with Parafilm® to reduce the entrapment of the alginate solution when immersed into the polymer solution.

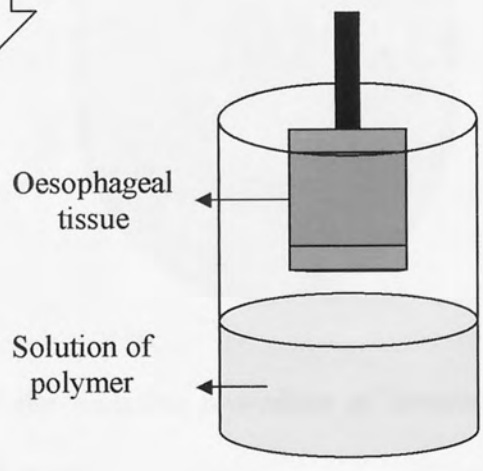
On the day of the experiment, the oesophageal tissue was cut into 4 cm length tubes and turned inside out, then stored in saline solution until required. The oesophageal tube was put onto the basket (after the basket was covered with Parafilm®). The basket was immersed into a beaker containing an alginate liquid and kept there for 30 seconds, it was then removed and held above the solution for about 2 minutes until the alginate liquid coating on the oesophageal tissue stopped dripping. Then the basket was immersed carefully into the vessel, whilst trying not to disturb the surface coating layer. Solution samples were collected at set time points for measurement. The alginate mass coated on the oesophageal tissue surface was calculated by the weight difference before and after the basket was immersed into the alginate liquid. The experimental procedure is schematically expressed in Figure 2.10.



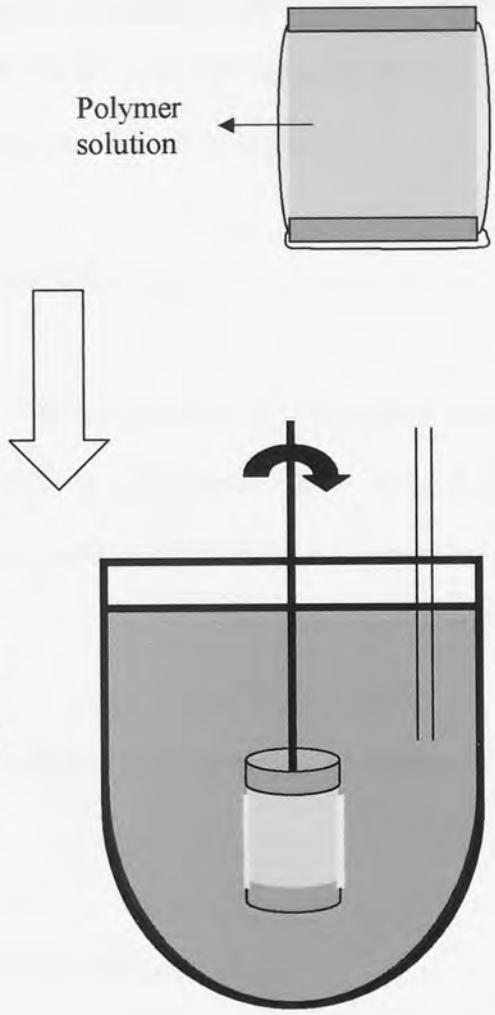
Oesophageal tube (epithelial surface facing out) covers outside of the basket. The flange on the bottom of the basket stops the tissue tube from slipping off.



The basket covered by tissue was immersed within the solution of polymer that was prepared previously by holding in a glass beaker. The weight of the beaker was noted before immersion.



The tissue was removed from the polymer solution whilst the beaker under it collected the solution drops from the tissue until equilibration. Then the weight of the beaker was reweighed to calculate the exact mass of polymer solution left on the tissue.



After the polymer solution coated the tissue it was immersed into the washing medium and the shaft started rotate at a set speed. The solution sample was withdrawn from the bulk liquid at designated time points for analysis.

Figure 2.10 Schematic representation of the retention procedure of bioadhesive solution using USP dissolution apparatus I as tool

The rotation speed was set at 60 rpm, which could be transferred to linear speed through equation 2.9.

$$V = \omega \bullet 2\pi r \qquad \text{Equation 2.9}$$

Where V is linear speed of the basket surface, ω is rotation speed 60 rpm, r is the radius of the basket 1.3 cm. Thus the surface linear speed was calculated and equal to

8.17 cm/sec. The calibration method was as same as described before. At each time point the concentration of the sample was calculated using calibration curve equation. This concentration multiplies with the whole volume of the solution 750 mL to get the percentage of the alginate solution off the tissue at each time point.

2.3.4 Different factors investigated that affect retention

Factors that may affect the retention of an alginate solution on oesophageal tissue were investigated using one or more of the labelling methods described. These studies are described in more detail in the following sections, divided by the analytical method used for the study.

2.3.4.1 Retention of fluorescein non-covalently labelled alginate solutions

- The effect of substrate on retention
- The effect of alginate concentration on retention
- The effect of alginate volume on retention
- The effect of washing medium pH on retention
- The effect of pepsin solution 0.1 % (w/v) as washing material on retention

2.3.4.2 Retention of fluorescently-modified beads labelled alginate solutions

- The effect of alginate concentration on retention
- The effect of bead surface charge on retention

2.3.4.3 Retention of radio labelled alginate solutions

- Different commercial products on retention
- The effect of different type of alginate on retention
- The effect of washing medium pH on retention

2.3.4.4 Dissolution apparatus I as retention tool

- The effect of alginate concentration on retention

Table 2.4 Factors that affected the retention of liquid alginates on porcine oesophagus

Factor	Label/method			
	Fluorescent label	Beads	Radiolabel	Dissolution method
Alginate concentration	yes	yes		yes
Alginate type	yes		yes	
Alginate volume	yes			
Different substrates	yes			
Buffer as washing media	yes		yes	
Pepsin solution as washing media	yes			
Beads surface charge		yes		
Commercial products			yes	

2.3.5 Methods of taking retention pictures

In this part, two labelling methods, fluorescein labelled alginate and fluorescently-modified beads dispersed within an alginate solution were used to visualise the layer on oesophageal tissue. The operation procedure for the two cases was identical.

2.3.5.1 Preparation of oesophageal tissue sections for microscopy

In the retention procedure at set time points (3 min, 12 min, 30 min) the oesophageal tissue was taken off the model and was frozen rapidly using liquid nitrogen or a freezing fixation spray agent. Once the tissue was frozen it was cut into small sections and embedded onto section holders. The embedding medium was purchased from Shandon, USA.

A cryo-sectioner (Bright OTF Cryostat) was used for sectioning the tissue. The frozen tissue was maintained in the instrument at a temperature of -20 °C. The sections were sliced to approximately 30 μm thicknesses. The temperature gradient between the warm slides and the cold sectioning knife enabled simple transfer of the sections onto the microscope slides.

2.3.5.2 Image analysis using fluorescence microscopy

The fluorescence images were viewed under x10 magnification and were taken under the dark background by a Nikon optical camera. The image analysis was calibrated according to a graticule image that was taken under the same conditions as the sample. A spatial calibration image was taken using the graticule to draw a line of known

length. This figure was set as a standard and the lines drawn were measured in reference to this value. All the other images were captured and the depth of the alginate layer on the top of the oesophageal surface was measured using this standard within the software package Image Tool 3.

In the fluorescent image study two types of pictures taken and analysed. The excitation wavelength used in the fluorescent microscopy was 530 nm.

- The retention of fluorescein labelled alginate solution at three set time points.
- The retention of fluorescently-modified beads labelled alginate solution at set time points.

2.4 RESULTS AND DISCUSSIONS

2.4.1 Retention of fluorescein non-covalently labelled alginate solution

2.4.1.1 The effect of substrates on retention

To compare the retention of different alginates solutions, three alginates (H120L 2 %, LF120 2 %, LFR5/60 5 % w/v) 0.1 mL were applied to three substrates; Parafilm®, hydrated dialysis membrane and porcine oesophageal tissue. The smooth surface of the Parafilm® made it a negative control for the membrane and tissue for bioadhesive properties. When hydrated, the surface of dialysis membrane becomes sticky; this property helps to mimic the surface of oesophageal tissue and improves the retention. The preparation of oesophageal tissue has been described in 2.2.1.1. The hydrated dialysis membrane was submerged in double distilled water overnight to remove the surface ions. It was then cut to size and used in the same way as oesophageal tissue.

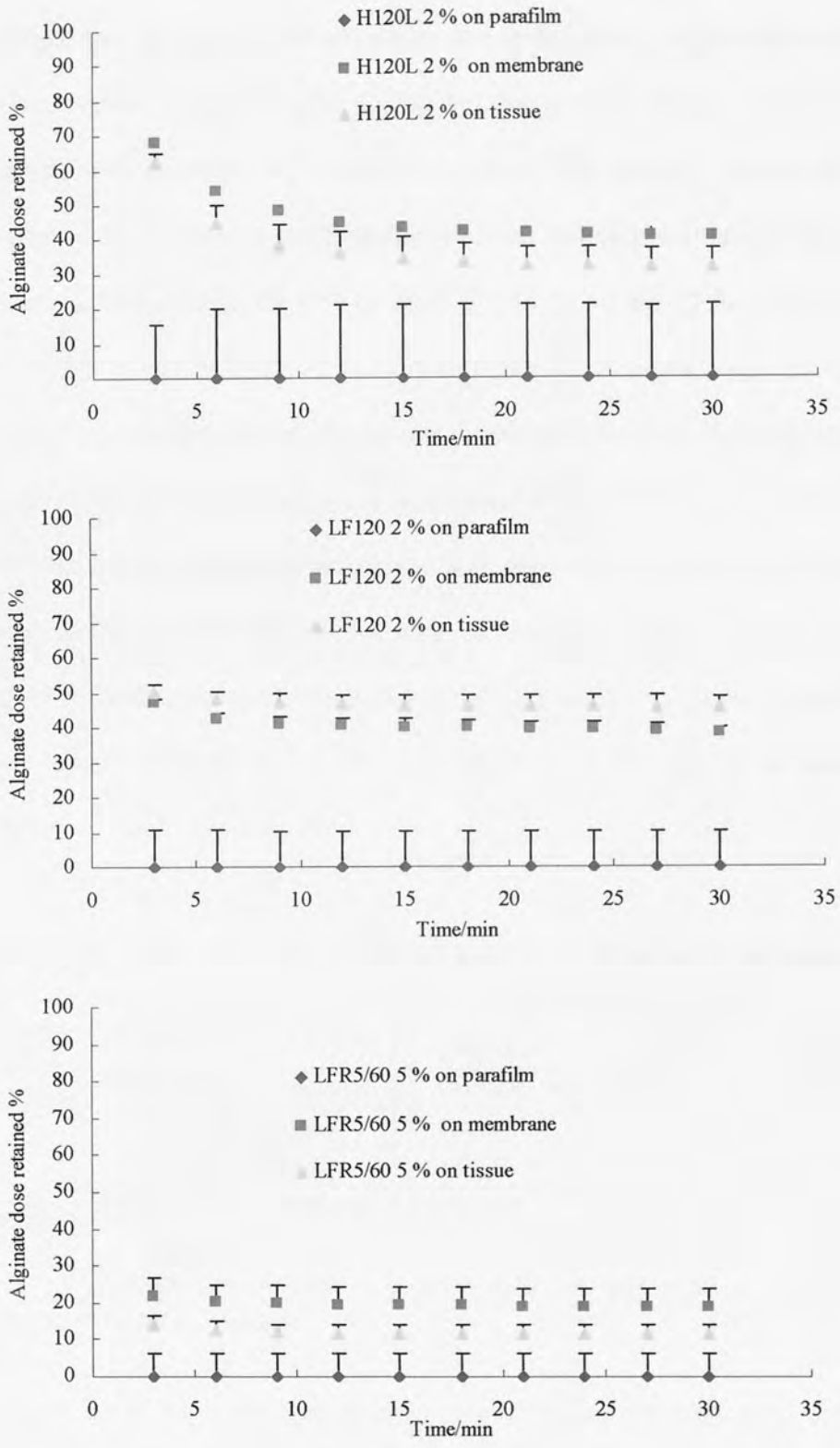


Figure 2.11 The retention of alginate solutions on three substrates, Parafilm®, dialysis membrane, porcine oesophageal tissue, measured using the retention model I. (mean + s.d., n=5)

Figure 2.11 shows the result of the three alginates washed off the three substrates. The Parafilm® has very small retention ability due to its smooth, impermeable surface. All the three alginate solutions applied on the Parafilm® were washed off (> 100 %), thus validating this substrate as a negative control. The dialysis membrane and the oesophageal tissue showed that alginates solutions had very similar retention. After 30 minutes washing 59.3 % ± 1.8 % vs. 68.1 % ± 5.2 % H120L (2 % w/v), 61.4% ± 1.2 % vs. 54.2 % ± 3.15 % LF120 (2 % w/v), 81.0 % ± 4.7 % vs. 88.2 % ± 2.3 % LFR5/60 (5 % w/v) was washed off the dialysis membrane and oesophageal tissue, respectively. Through ANOVA analysis at two time points 3rd and 30th minute (Table 2.5), the results indicated that dialysis membrane and oesophageal tissue had no significant difference, which means the two materials as biological substrates have very similar adhesive properties for the three alginates. For this similar adhesive property dialysis membrane can replace the oesophageal tissue in some studies to simplify the procedure.

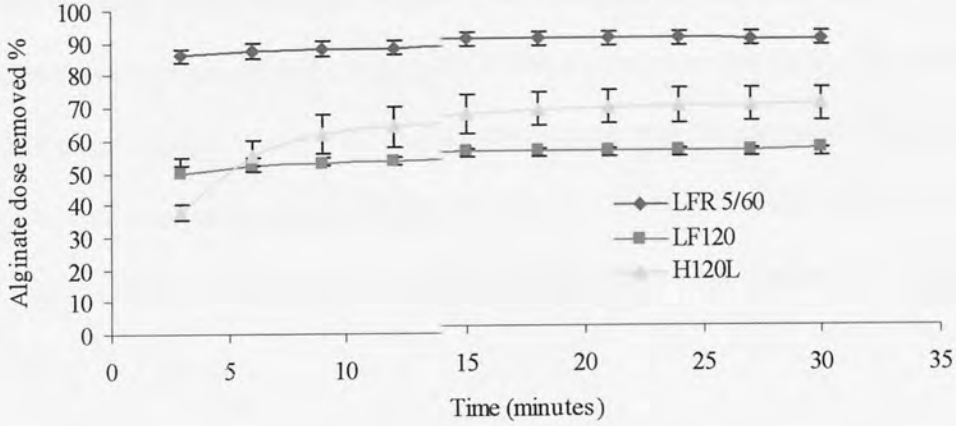
Table 2.5 ANOVA analysis of retention of alginate on three biological substrates

Time point	Comparison of 3 substrates	P value		
		LFR5/60	LF120	H120L
3 minute	Parafilm® vs membrane	P<0.001	P<0.001	P<0.001
	Parafilm® vs tissue	P<0.001	P<0.001	P<0.001
	membrane vs tissue	ns P> 0.05	ns P> 0.05	ns P> 0.05
30 minute	Parafilm® vs membrane	P<0.001	P<0.001	P<0.01
	Parafilm® vs tissue	P<0.001	P<0.001	P<0.01
	membrane vs tissue	ns P> 0.05	ns P> 0.05	ns P>0.05

ns = no significant difference

For comparison, the retention of the three alginates on both hydrated dialysis membrane and oesophageal tissue are shown in the figure 2.12.

A.



B.

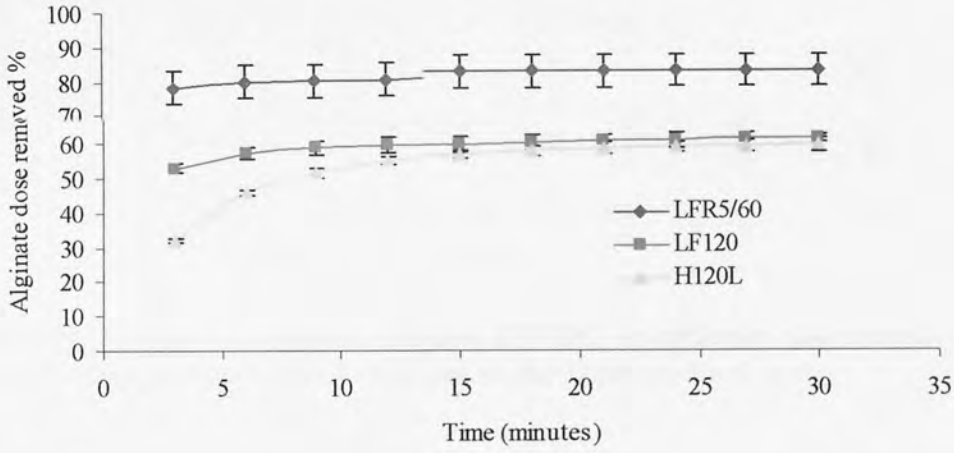


Figure 2.12 Comparison of the retention of three alginates on biological substrates using retention model I (H120L 2 %, LF120 2 %, LFR5/60 5 % w/v) A. On porcine oesophageal tissue B. On hydrated dialysis membrane (mean ± s.d, n=5)

Figure 2.12 indicates that LFR5/60 had the least retention ability. Until 9 to 12 minutes, H120L showed greater retention than LF120 yet after that time both of alginates had very similar percentage of dosage washed off the substrates.

2.4.1.2 The effect of alginate concentration on retention

The above study used retention model I; in changing to retention model II slight differences were observed in the retention of the alginates in this study. The retention of the alginate solutions with different concentrations was investigated. Two sodium alginates were used in this study. H120L at 1 %, 2 %, 3 %, 4 % (w/v) and LF120 at 1 %, 2 %, 4 % (w/v) were applied and the results are shown on Figures 2.13 and 2.14, respectively.

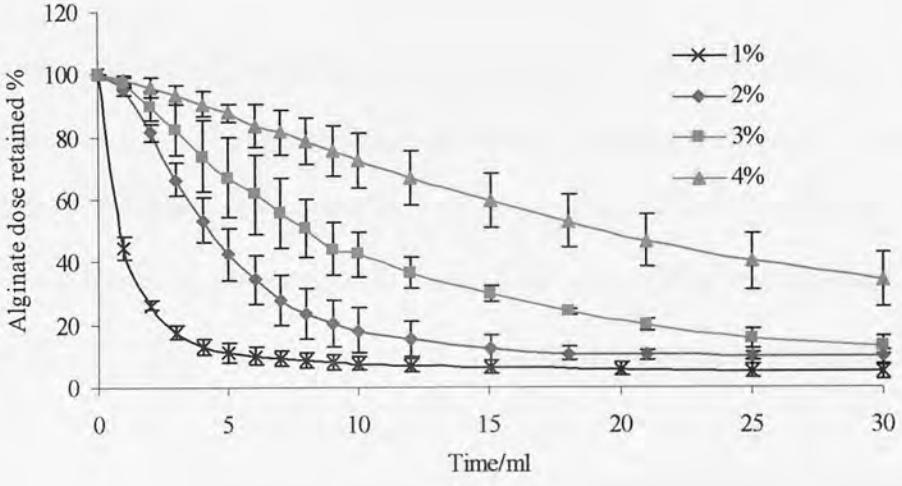


Figure 2.13 Retention of alginate solution (H120L) at different concentrations on porcine oesophageal tissue using retention model II (mean ± s.d, n=5)

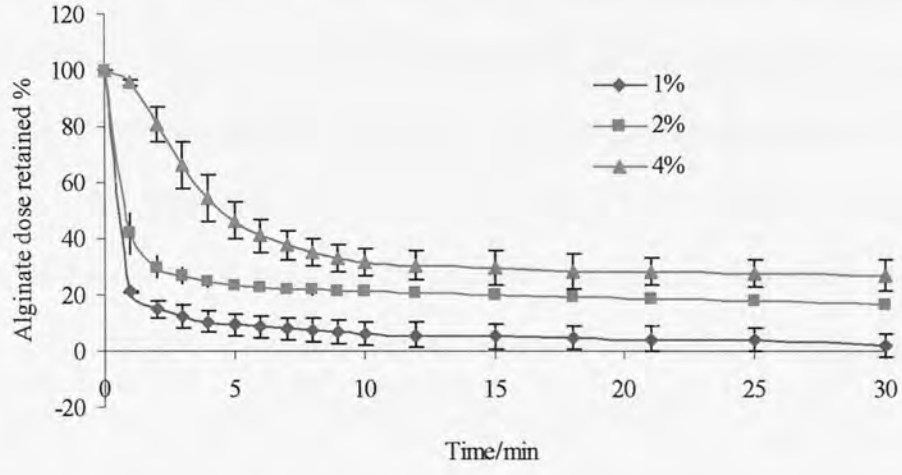


Figure 2.14 Retention of alginate solution (LF120) at different concentrations on porcine oesophageal tissue using retention model II (mean ± s.d, n=5)

ANOVA analysis indicated that at 30 minutes the difference in retention between the different concentrations of each alginate was significant for each value.

As the concentration of alginate increased, an associated increase in viscosity was observed, increased viscosity slows down the flowing speed of the alginate solution. In next chapter the viscosity of different concentrations is measured.

2.4.1.3 Volume of alginate solutions

In most retention experiments the same volume of alginate solution (1 mL) was dispersed on the substrate. In this study, different volumes of alginate solutions were applied on the tissue surface and their effect on retention was investigated. LF120 2 % (w/v) at a range of volume 0.5 mL, 1 mL, 2 mL was applied on tissue and washed by distilled water at the speed of 1 mL/min. The results are plotted in Figure 2.15.

With a volume of 0.5 mL, 72.3 % $\pm$ 1.9 % was washed off at 30 minutes, meaning about 0.14 mL was retained on the tissue after 30 minute washing. With a volume of 1 mL 66.1 % $\pm$ 2.8 % washed off, around 0.34 mL was retained at 30 minutes and with 2 mL 65.2 % $\pm$ 1.3 % was washed off, around 0.69 mL was retained after half an hour. The results demonstrated that when the volume of applied alginate solution was less than 1mL, the percentage of solution retained on tissue was less than that when applied as a volume of more than 1mL. And when the volume was increased to 1 mL and 2 mL, the percentage retained was similar. However the volume of alginate retained on the tissue surface increased as the dose volume increased.

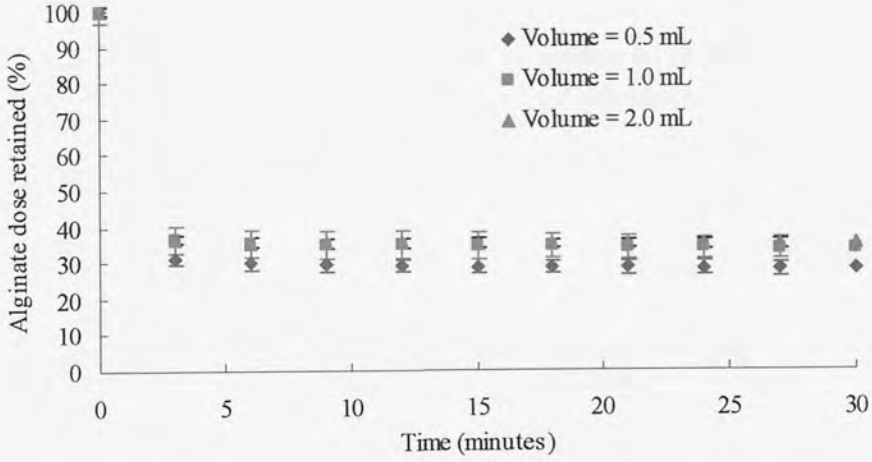


Figure 2.15 Retention comparison of different volumes of alginate solution (LF120 2 % w/v) applied on porcine oesophageal tissue using retention model I (mean ± s.d., n=5)

In the study of volume comparison retention model I was used, the eluate collection points were set at 3 minute intervals and the calculation method was similar to that used in the study of different substrate comparisons.

2.4.1.4 Pepsin solution as a washing material

Pepsin, one of the main damaging factors to oesophageal tissue in gastric reflux, was investigated as a washing medium to investigate the effect on the retention of alginate.

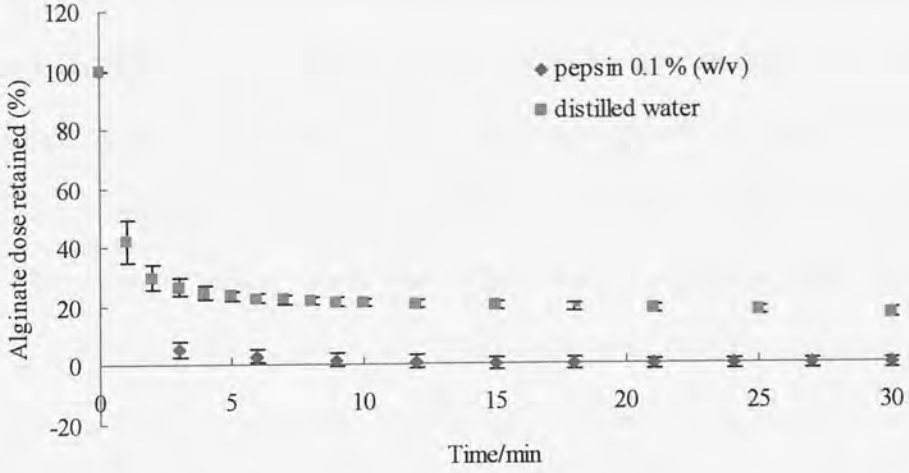


Figure 2.16 Retention of alginate solution (LF120 2 % w/v) washed by medium, 0.1 % pepsin solution or distilled water using retention model II (mean ± s.d., n=5)

Pepsin A bought from Sigma was made up to a solution of approximate physiological concentration 0.1 % (w/v) as a washing medium within double distilled water. Alginate LF120 2 % (w/v) was used in this experiment. The retention result is shown in Figure 2.16. 101.6 % ± 1.9 % alginate was washed off by the pepsin solution within 30 minutes. 83.1 % ± 1.5 % was washed off by the control, distilled water at 30 minutes. This data indicated that the pepsin solution decreased the retention of the alginate solution. The next chapter, "Viscosity study" demonstrates that the pepsin reduced the viscosity of alginate solution, which may explain why pepsin solution increased the percentage of the alginate washed off the tissue.

2.4.1.5 Buffers with different pH as washing materials

In GORD and other acid related oesophageal diseases, acidic gastric reflux is a main reason for damage observed within the oesophagus. Much of the retained acidic reflux on the surface of oesophagus is removed by the oesophageal physiological peristaltic

wave and swallowed saliva washing the surface, yet a low pH can persist on the oesophageal surface. Alginate forms a gel at low pH values. In this study, a range of buffer solutions were made at pH 4, 6, 8 as washing materials to observe their effects on the retention of alginate. A 2 % (w/v) LF120 1 mL dose was applied to oesophageal tissue. The retention results are shown in Figure 2.17. When alginate liquid was dispersed into pH 2 buffer, a hard gel formed that was difficult to measure and a calibration could not be produced, therefore pH 2 buffer was not used as a washing medium within this study.

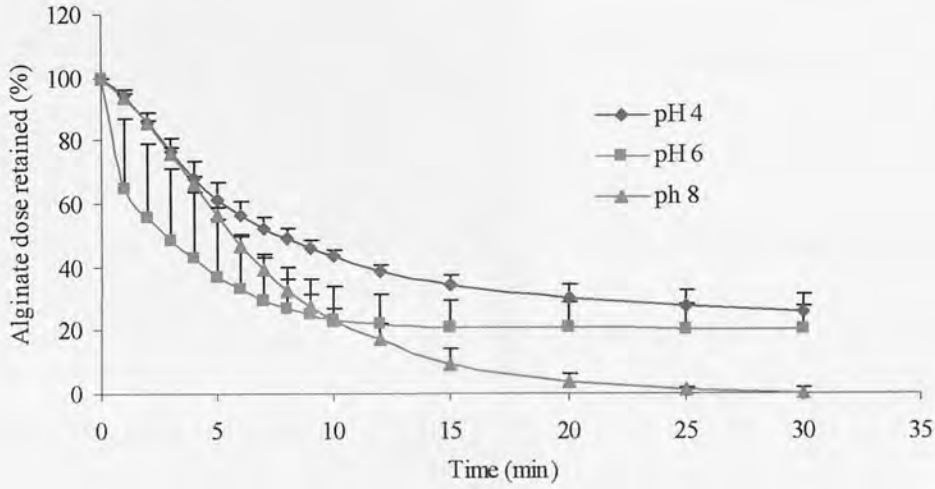
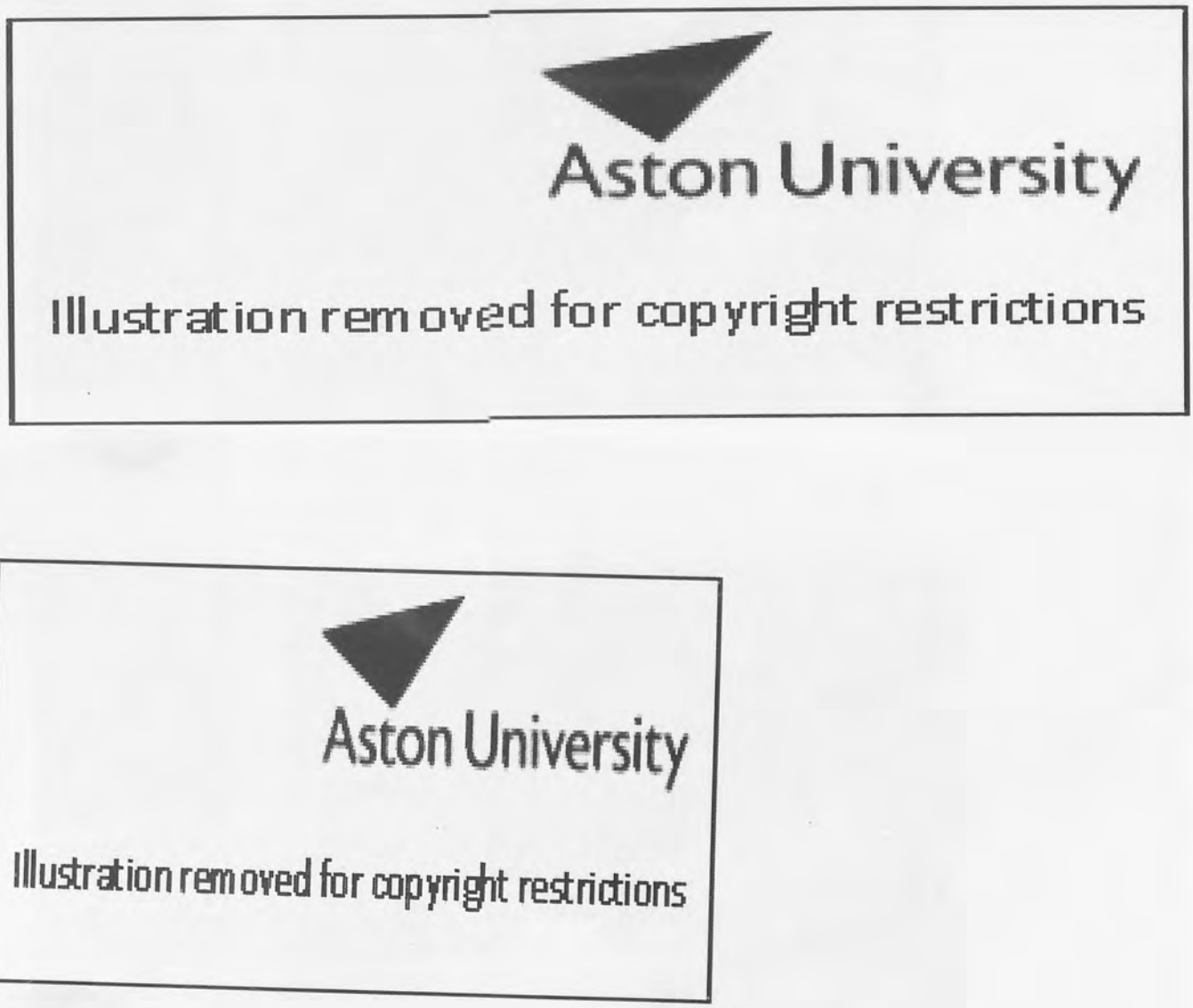


Figure 2.17 Retention of alginate solution (LF120 2 % w/v) washed by buffers at pH 4, 6, 8 using retention model II (mean + s.d., n=5)

After 30 minutes, the retained dosage percentage was 25.9 % $\pm$ 5.7 % with pH 4 buffer, 19.9 % $\pm$ 7.8 % with pH 6 buffer, 0.22 % $\pm$ 1.52 % with pH 8 buffer, which indicated that the lower pH will increase the retention of alginate solution. This may be because of the increased viscosity of the alginate solution at low pH. A similar experiment was performed in radio label experiments (section 2.4.3.3).

Figure 2.18 Images of fluorescently labelled H120L 2 % (w/v) on porcine oesophageal tissue surface at 3 time points (A. 3 minute; B. 12 minute; C. 30 minute) Calibration bar represents 0.1mm

For each image one piece of oesophageal tissue was used. At a set time point the tissue was taken off the model and handled through the procedure of tissue embedding, freezing, sectioning, and image capture that was described in detail in 2.3.6.



B. 12 minutes

Figure 2.19 Images of labelled LF120 2 % (w/v) as protective layer on tissue surface at 2 time points, left images taken under fluorescence, right images taken under normal light microscopy

The pictures were taken under x 10 magnification. The fluorescent labelled alginates were used; H120L 2 % w/v, LF120 2 % w/v, and LFR5/60 5 % w/v. Because of the weak adhesive ability of LFR5/60, labelled LFR5/60 layer could not be viewed under the microscope. Figure 2.18 and 2.19 show the pictures taken at set times points. The depths of the alginate layer on the top of the tissue surface in the images were measured using Image Tool 3. The results are in Table 2.6.

Table 2.6 Depth of labelled alginates on oesophageal surface (n=6)

Time	H120L 2 % (w/v)	LF120 2 % (w/v)	LFR5/60 5 % (w/v)
	Depth /mm	Depth /mm	Depth /mm
3 min	0.53 ± 0.04	0.07 ± 0.01	n/a
12 min	0.47 ± 0.05	0.02 ± 0.01	n/a
30 min	0.30 ± 0.04	0	n/a

Table 2.6 shows that the H120L 2 % (w/v) coated with the deepest layer on the oesophageal surface in the first 3 minutes among the three alginates, which was associated with the retention results, H120L 2 % (w/v) had the best retention in the first few minutes. For the low viscosity of LFR5/60 5 % (w/v) even within the first 3 minutes little liquid LFR5/60 was shown on the oesophageal surface. This result correlates with the retention results.

2.4.2 Retention of fluorescently-modified beads labelled alginate solution

Alginate solution cannot only act as a protective layer on oesophageal tissue, but is also a promising drug delivery system. In this study, the potential of liquid alginate as drug delivery system was investigated.

2.4.2.1 Concentration of alginate solution

Because of the fluorescent properties of the modified beads, the beads were used as a label investigating the retention of alginate solutions. In this experiment LFR5/60 of different concentrations (2 %, 5 %, 10 %, w/v) was labelled with carboxylate beads. The 2 % (w/v) alginate was entirely washed off the tissue within just two minutes, at 5 % (w/v) the alginate was retained on tissue for up to 10 minutes and 10 % (w/v) LFR5/60 was retained on tissue up to 20 minutes (Figure 2.20).

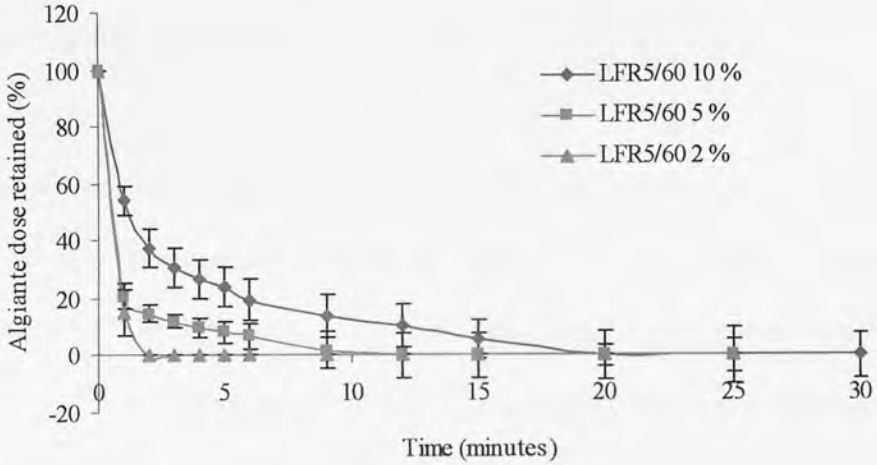


Figure 2.20 Retention of fluorescently-modified carboxylate beads label LFR5/60 (2 %, 5 %, 10 % w/v) using retention model II (mean ± s.d., n=5)

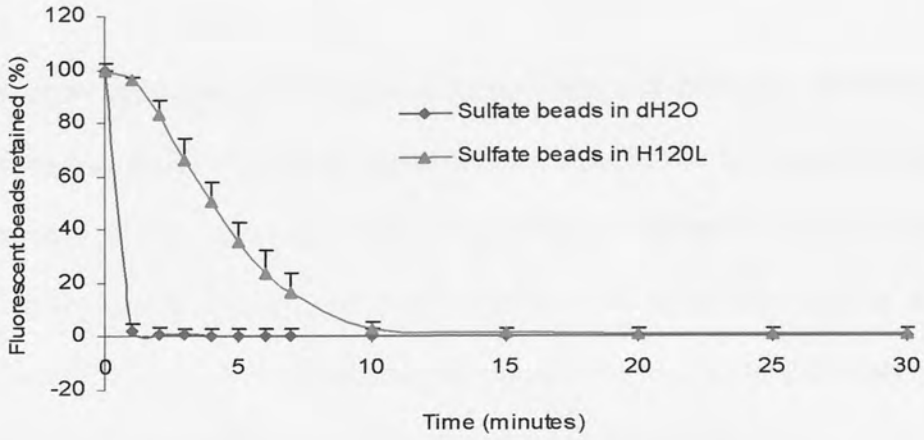
The experimental result indicated the same trend as the concentration study in 2.4.1.2 that is, when alginate concentration increased, the retention increased as well. The reason may be that increasing concentration leads to an increase in the viscosity of the solution so that retention becomes prolonged.

2.4.2.2 A comparison of the retention of model drugs on oesophageal tissue: solutions of sodium alginate versus water as a control

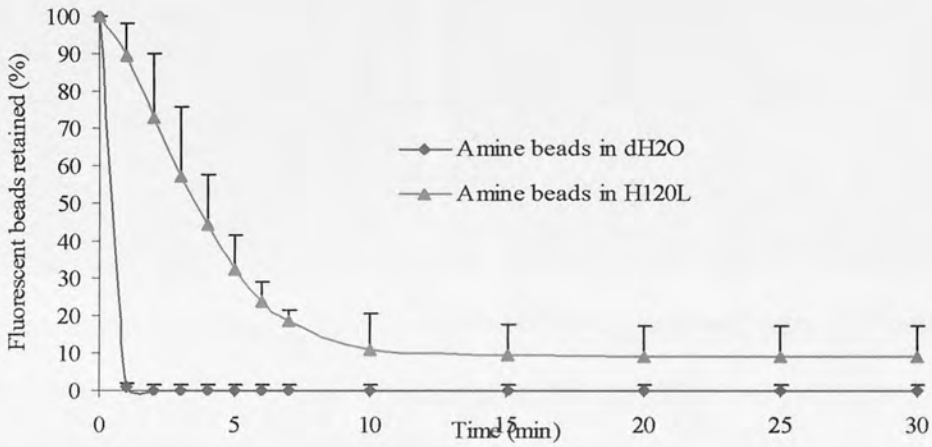
Three fluorescently-modified beads, sulfate, carboxylic, amine (Sigma) were used to mimic drug particles. The diameter of the beads is approximately 2μm. Sulfate beads have bivalent negative charges, carboxylic beads have a negative charge, and amine beads have a positive charge. 1mL bead suspension was diluted into 50 mL distilled water. Each bead suspension was made up to 2 % w/v alginate solution to compare with the beads in distilled water (without alginate). The alginate solution used in this study was H120L 2 % (w/v).

The results of this study are shown in Figure 2.21. All of the modified beads in distilled water were washed off tissue within less than five minutes. The percentage retained on the tissue after 30 minutes washing was 1.2 % $\pm$ 1.8 %, 9.14 $\pm$ 8.13 %, 6.69 % $\pm$ 2.19 %, of the three modified beads; sulfate, amine and carboxylate mixed with H120L 2 %. This difference in percentage of the three beads retained on tissue can be explained by the fact that saliva and mucin on the oesophageal surface is negatively charged. Sulfate beads with a bivalent negative charges were repelled the most by surface mucin, but carboxylate with one negative charge had a weaker repelling force, so more was retained and amine beads with a positive charge were attracted by mucin molecules so were retained the most on the tissue. Without alginate as a bead carrier, all three controls (beads suspended in distilled water) were only retained on the tissue for 2 or 3 minutes before they were all washed off. The above results demonstrated that the viscous alginate carrier significantly increased the retention of modified beads compared to an aqueous suspension ($P < 0.05$, ANOVA).

A.



B.



C.

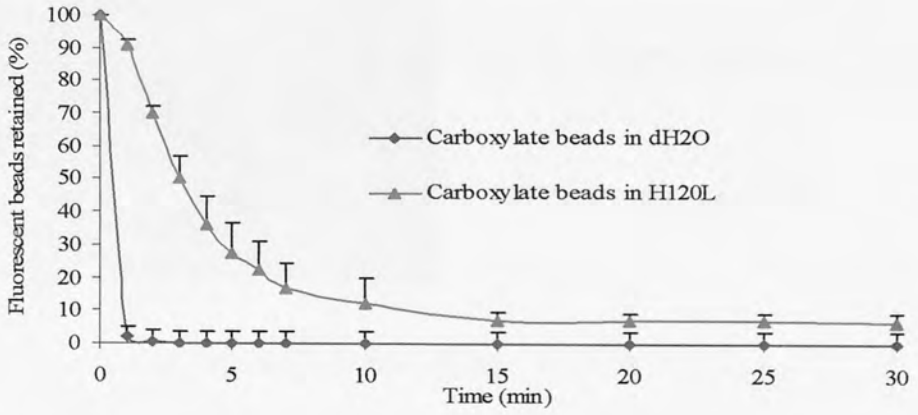


Figure 2.21 Retention comparison of beads suspension with and without alginate H120L 2 % w/v, measured using retention modle II (A. Sulfate, B. Amine, C. Carboxylate, beads mean ± s.d., n=5)

2.4.2.3 Imagines showing the alginate solution as drug delivery tool

The retention experiment investigated the possibility and capability of the alginate solution as a drug delivery system targeted to the oesophagus. Images collected via a microscope, directly present the retention of beads in alginate solutions at set time points. Three modified beads that mimic drug particles were suspended in alginate solutions (H120L 2 % w/v). The pictures shown are: Figure 2.22 (Carboxylate beads); Figure 2.23 (Amine beads); Figure 2.24 (Sulfate beads) at set time points.

B. Image of beads suspended within H120 2 % w/v at 12 minutes

Figure 2.22 Images of modified carboxylic beads in an alginate carrier (A. 3 min; B. 12 min. Calibration bar represents 100 micrometer)

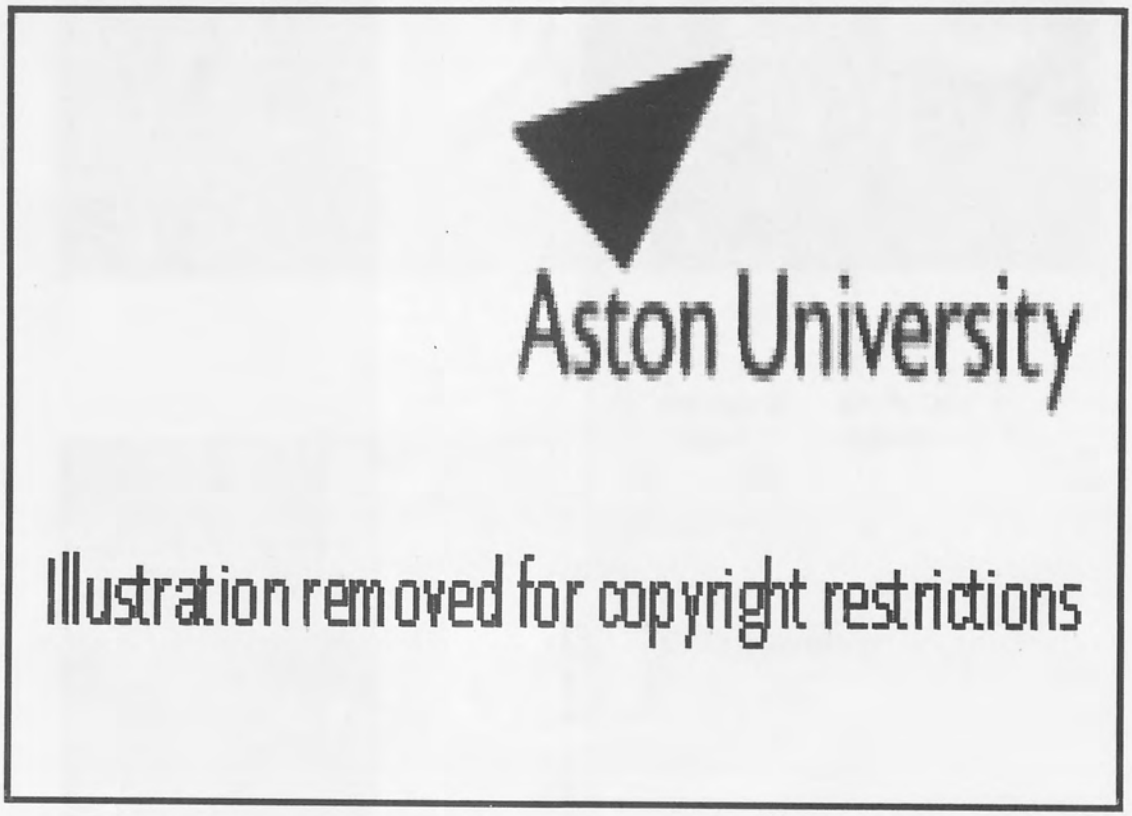


Figure 2.23 Images of modified amine beads in an alginate carrier (H120L 2% w/v) at three time points (A. 3 min; B. 12 min; C. 30 min. Calibration bar represents 100 micrometers)

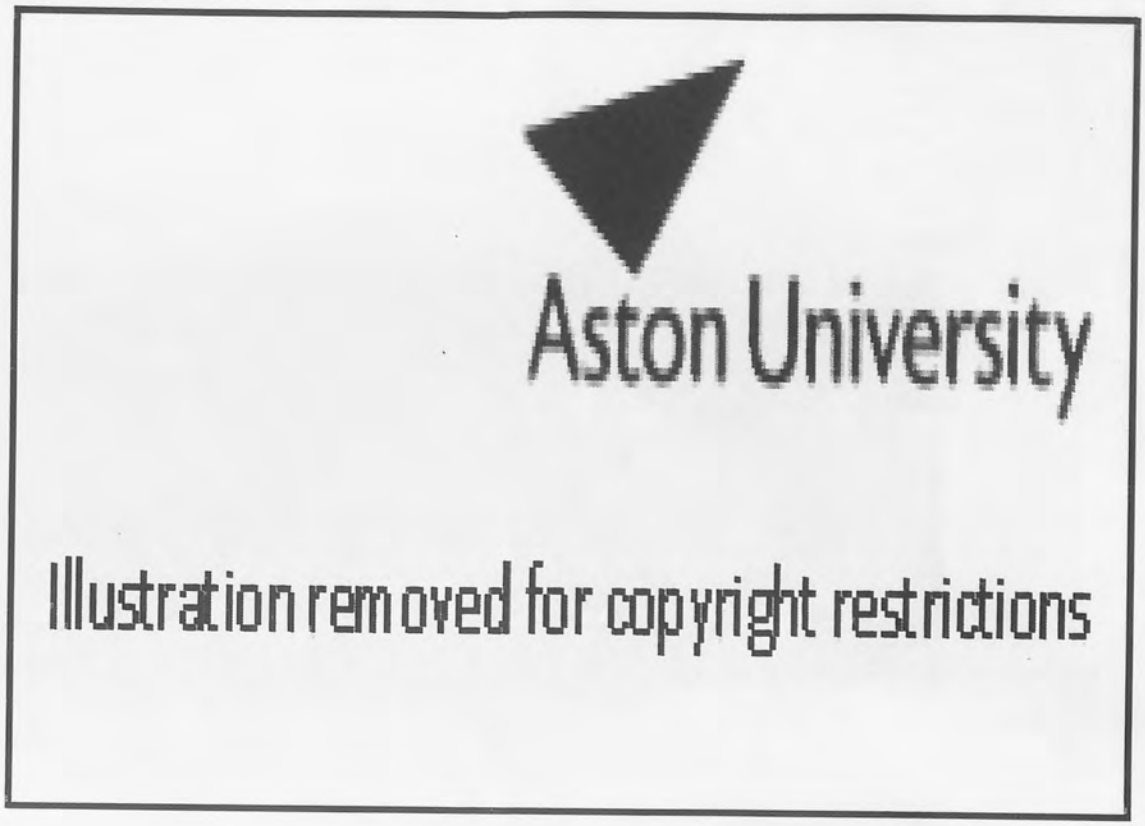


Figure 2.24 Images of modified sulfate beads in an alginate carrier (left; H120L 2 % w/v and right; LF120 2%, w/v), A. 3 min; B. 12 min; C. 30 min. (calibration bar represents 100 micrometer)

All the images were captured under the same conditions. The variance in the oesophageal tissue and the uneven distribution of the alginate over the tissue surface meant that measurements were not determined at all conditions. For example carboxylate beads in H120L at 30 minutes were not measurable. The depth of the alginate layer retained on the tissue at set times was calculated using Image Tool 3 and the results are presented in Table 2.7.

Table 2.7 Depth of alginate (H120L) layer mixed with modified fluorescence beads on oesophageal surface (mean ± s.d., n=6)

	COOH	NH_2	SO_4
Time	Depth /mm	Depth /mm	Depth /mm
3 min	0.45 ± 0.04	0.24 ± 0.02	0.29 ± 0.02
12 min	0.12 ± 0.02	0.11 ± 0.01	0.16 ± 0.02
30 min	N/A	0.04 ± 0.01	0

Table 2.7 shows the depth of the alginate carrier mixed with 3 modified fluorescent beads at three specified time points. The different depths of the three mixed systems were maybe due to the surface charges of the beads. Positive charges associated with amine particles demonstrated a thinner adhered layer compared to the negatively charged beads. The positively charged beads may interact with the negatively charged alginate leading to a complexation that increased the density of the alginate on the tissue thus a thinner layer was observed. However the negative charges on the surface of sulfate and carboxylate particles may disperse more homogeneously throughout the alginate solution to slightly increase the thickness of the adhered layers of the two beads on oesophagus. Overall the same conclusions as in the retention study can be

made; viscous alginate increased the retention of modified beads indicating that alginate would enhance the delivery of such particles to the oesophagus.

2.4.3 Retention of radio-labelled alginate solutions

The fluorescent spectrophotometer is limited and cannot measure cloudy solutions therefore, radiochemical Technetium ($^{99}Tc_m$) was introduced as an alternative labelling material. The retention of alginate-based commercial products (Gaviscon Advance®, Gaviscon Liquid®, and Gaviscon Mikstur®) were measured. When mucin was the washing material, the elute collected was cloudy thus these experiments required a non-colorimetric assay. The alginate solution used was labelled with $^{99}Tc_m$. A comparison of the retention of fluorescently labelled alginates with the alginates (H120L 2 %, LF120 2 %, LFR5/60 5 % w/v) labelled with radiochemical was investigated. The experimental procedure and radio safety operation rules for these experiments were described in detail in 2.3.3.

2.4.3.1 Retention comparison of three Gaviscon® commercial products

In Figure 2.25 the retention curves of the three alginate-based products are shown over 30 minutes. After the set period of washing 2.94 % $\pm$ 1.81 % of Gaviscon Advance®, 1.05 % $\pm$ 3.86 % of Gaviscon Liquid®, 2.21 % $\pm$ 1.45 % of Gaviscon Mikstur® was retained on the tissue, respectively. Because the main ingredient in Gaviscon® is LFR5/60 (Gaviscon Advance® 10 % w/v, Gaviscon Liquid® 5 %w/v and Gaviscon Mikstur® 5.5 % w/v), the percentage retained on tissue was lower than expected after 30 minutes washing. Within first 5 minutes, Gaviscon Advance® showed the better retention than the other two formulations. In fluorescently labelled retention, LFR5/60

at 5 % was retained on tissue for up to 10 minutes and LFR5/60 at 10 % was retained on the tissue for up to 20 minutes. The slight increase in retention of the alginate-based products after 30 minutes washing compared to that of pure alginate LFR5/60 may be caused by the additional excipients present within the commercial formulations.

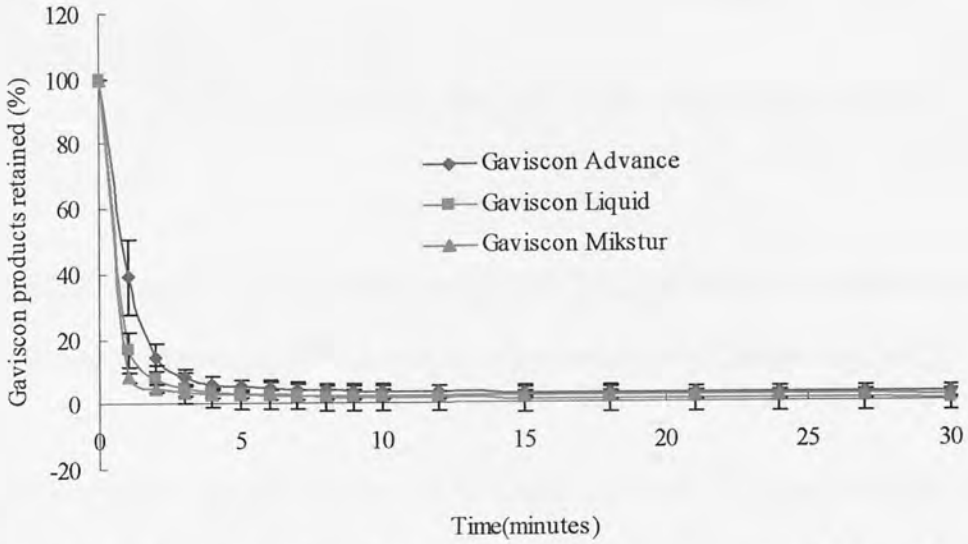


Figure 2.25 Retention of radio labelled Gaviscon® commercial products on oesophageal tissue using retention model II (mean ± s.d., n=5)

2.4.3.2 Retention of three alginate solutions compared with fluorescein labelled alginate solutions

A direct comparison of the retention of the alginate liquids using the fluorescent dye and the radiolabel was performed. Figure 2.26 shows the retention curves of the alginate solutions. After half an hour, the percentage of H120L 2 % (w/v), LF120 2 % (w/v), LFR 5 % (w/v) retained on the tissue was 6.68 % $\pm$ 1.74 %, 3.31 % $\pm$ 1.52 % and 3.13 % $\pm$ 1.82 %, respectively using the radiolabel.

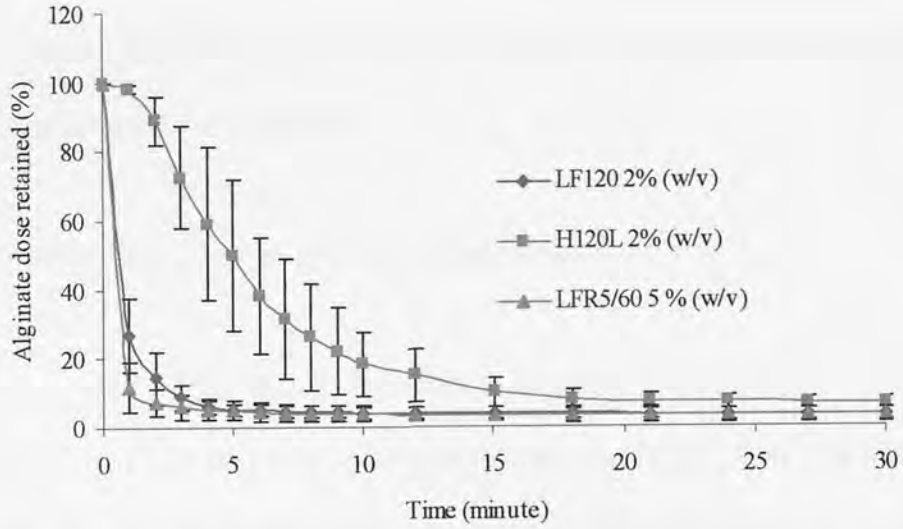


Figure 2.26 Retention of three alginate (H120L 2 %, LF120 2 %, LFR5/60 5 % w/v) labelled with radiochemical $^{99}Tc_m$ using retention model II (mean ± sd., n=5)

The data in Figure 2.26 shows that 3.13 % ± 1.82 % of LFR at 5 %w/v was retained on the tissue, which is quite similar to the two commercial alginate-based products (Gaviscon Liquid® 1.05 % ± 3.86 % and Gaviscon Mikstur® 2.21 % ± 1.45 %). But the other two alginates showed retention values that were quite different from the fluorescent labelled retention. H120L 2 % and LF120 2 % was 6.68 % ± 1.74 % and 3.31 % ± 1.52 % (radio labelled) retained versus 16.9 % ± 1.45 % and 9.6 % ± 1.9 % (fluorescent label), respectively. This big difference may be caused by several factors. Firstly $^{99}Tc_m$ solution as a label was added to alginate solution at a concentration of 0.1 mL/1.5 mL, which may reduce the viscosity that can reduce the adhesive ability. The viscosity was not measurable due to the very small quantities of radiolabel purchased combined with the large volume size required for viscosity testing using the Brookfield viscometer. Secondly, the fluorescent dye may diffuse into and remain associated within the oesophageal epithelial layer whereas this would not occur for the technetium linked to the colloid carrier. Finally the fluorescence spectrophotometer

was limited in its ability to read cloudy solutions, the slightly brown colour of alginate solution may slightly reduce the fluorescence reading that will increase the calculation of the percentage of retained alginate.

2.4.3.3 Buffers with different pH as washing materials

Buffers at a range of pH values were used as washing materials. After 30 minutes washing by buffer solutions, the percentage of alginate H120L 2 % retained on the tissue was 21.43 % ± 6.42 % at pH 4; 9.61 % ± 0.51 % at pH 6, 10.46 % ± 1.47 % at pH 8. The three curve trends are as expected, lower pH has higher retention and high pH has lower retention. This is also in agreement with the data collected using the fluorescent label.

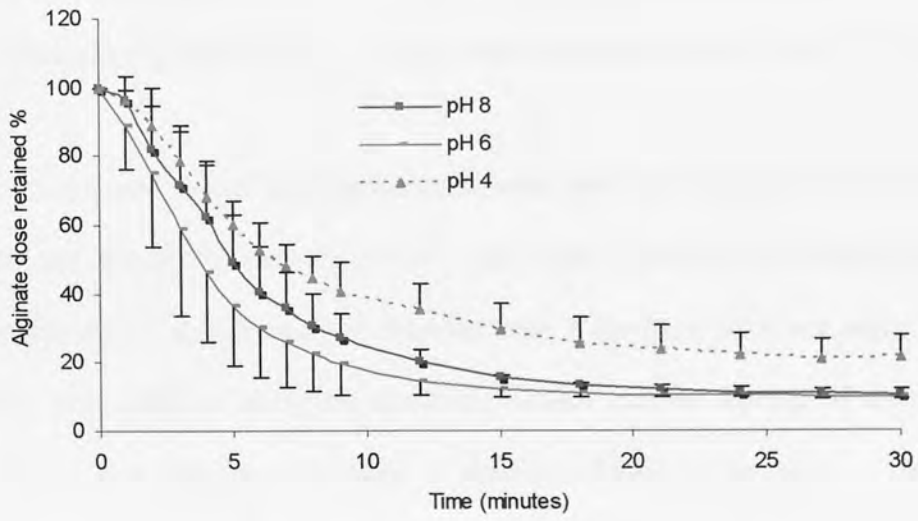


Figure 2.27 Retention of alginate solutions when buffers were used as a washing materials, using retention model II (mean ± s.d., n=5)

2.4.4 Retention using dissolution apparatus I as tool

The experimental method was described in 2.3.4. H120L at different concentrations; 1 %, 2 %, 4 % (w/v) was used in this study. The result retention curves are shown in Figure 2.28.

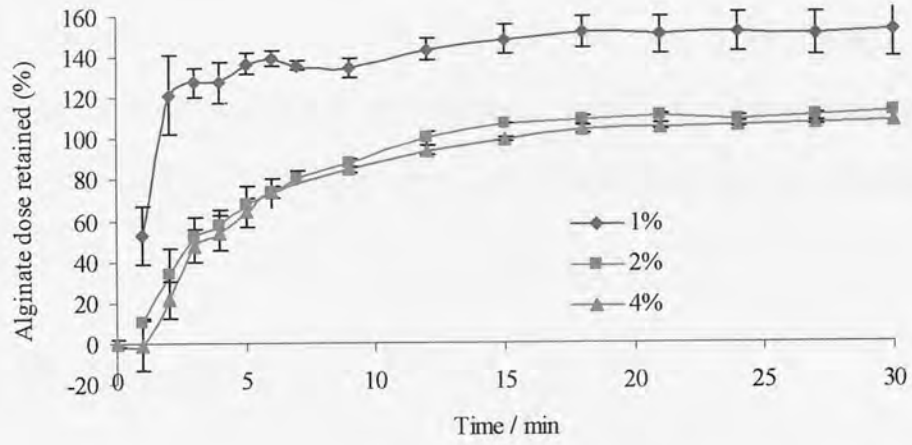


Figure 2.28 Retention of alginate solution (H120L) at different concentration using USP dissolution apparatus I as experimental model (mean ± s.d., n=5)

During this experimental procedure the alginate was removed from the outer tissue surface and dissolution occurred slowly over time. Samples were taken from the dissolution media at a distance of approximately 2 cm from the tissue surface; these samples were analysed using the spectrophotometer and the amount of fluorescence was used to calculate the percentage of alginate adhered to the tissue surface. The results above show values greater than 100 %; this is thought to be due to the fact that the bulk media was not homogenous and that the fluorescein associated with the alginate did not dissolve immediately into a homogeneous solution but formed a more concentrated layer near the surface of the oesophageal tissue. Sampling from within 2 cm of the tissue surface meant that upon converting this value to the percentage

retained an underestimation was produced thus explaining the data above where greater than 100 % has been removed. With the higher concentrations this effect would be larger thus it is likely that a greater percentage of the applied alginate remained at the time points measured.

To make the data comparable, all the solution samples collected from the vessel were from a fixed location. So the results do not show the exact percentage of applied dosage washed off oesophagus but suggested a trend; when concentration of alginate (H120L) increased, the retention increased, which is same trend as the other methods presented.

2.5 CONCLUSIONS

Different labelling methods were used in this study, yet they all showed similar trends in alginate retention. Both the fluorescent label and the radio-label suggested that H120L gave the best retention of the three alginates at the same concentration, especially within the first 12 minutes. Fluorescent images showed similar results that at 3 and 12 minutes the depths of the alginate H120L was greatest on the oesophageal surface. LFR5/60 showed the least retention. Using different pH buffers as washing material demonstrated similar results with both fluorescent and radio labelling methods; that lower pH buffer (pH 4) increased the alginate retention compared to higher pH buffers (pH 6 and 8). In the study of concentration effect on retention, the fluorescent label method indicated that for both alginates H120L and LF120 increasing concentrations significantly improved the retention. LFR5/60 at 3 concentrations showed the same trend; 2 % LFR5/60 was only retained on the tissue for 3 minutes, whereas 5 % and 10 % w/v coated the tissue for 10 and 20 minutes, respectively.

Gastric pepsin, as expected, decreased the alginate retention significantly, which was confirmed by the retention of fluorescent labelled LF120 2 % w/v. This study also noted that sodium alginate liquids have the potential to be an adhesive drug carrier for treating local oesophageal diseases as it can extend drug retention within oesophagus.

This has been published by Batchelor et al: Feasibility of a bioadhesive drug delivery system targeted to oesophageal tissue. (2004) European Journal of Pharmaceutics and Biopharmaceutics 57: 295-298

All of the above experimental results suggested that sodium alginates can form a bioadhesive coating on the oesophageal surface to protect the tissue from damage caused by gastric reflux content.

CHAPTER 3 VISCOSITY STUDY

3.1 INTRODUCTION

3.1.1 Theory of viscosity

Rheology is defined by Webster's Dictionary as "the study of the change in form and the flow of matter, embracing elasticity, viscosity, and plasticity". Because of the simple measurement method for viscosity compared to other rheological characteristics of a material, viscosity becomes a valuable tool for analysis. Viscosity is described as resistance to flow or movement, further defined as internal friction of a fluid, caused by molecular attraction (Aulton, 1988). The friction becomes apparent when a layer of fluid moves over another layer. The greater the friction, the greater the force required for this movement, which is called "shear".

Consider a cube of fluid where a force is applied on the uppermost layer, the force applied is termed shear force, F, the force causes the fluid cube to move at a different velocity at the different layers from the top to the bottom. The movement velocity of the surface layer is divided by the height of the fluid block that is defined as shear rate D with a unit of reciprocal second (s^{-1}). Newton expressed the viscosity η as:

$$\eta = F/D \qquad 3.1$$

Viscosity η has a foundation unit "poise" or p that could be transferred to International System units of Pascal-second (Pa.s) or milli- Pascal-second (mPa.s). 1 Pa.s = 1 p or 1 mPa.s = 1cp (centipoise).

Newtonian fluids show very stable flow behaviour, as the viscosity does not change with shear rate. Typical Newtonian fluids include water and thin motor oils. Their flow behaviour follows Newton's law (3.1). However, most fluids show more complex flow behaviour called non-Newtonian. The types of non-Newtonian flow behaviour are characterised by the viscosity change in response to variations of shear rate, including pseudoplastic, dilatant and plastic flows.

Pseudoplastic fluids display decreasing viscosity with an increasing shear rate, probably the most common of the non-Newtonian fluids include paints or emulsions. Aqueous dispersions of sodium alginates exhibit this type of flow (pseudoplastic). Viscosity increases with increasing shear rate characterise a *dilatant fluid*, such as clay slurries, corn starch in water and sand/water mixtures. *Plastic fluids* have a yield value where only the force applied on the fluid beyond this value will induce flow. Finally plastic fluids may display Newtonian, pseudoplastic, or dilatant characteristics.

The above three flow behaviours assume that the viscosity of a fluid only varies with shear rate, independent of the length of the time that the shear rate is applied. But this only happens in an ideal situation. Many non-Newtonian materials are colloidal and the flowing elements, such as particles or macromolecules, may not immediately adapt to the shearing conditions, thus when the fluid is subjected to constant shear rate the viscosity decreases with time, which is termed *thixotropy (or shear thinning)*. If the viscosity of the fluid increases with time at the same shear rate this is defined as

rheopexy (or shear thickening). Both characteristics are due to time-dependent behaviour of fluids.

The viscosity of a non-Newtonian fluid is affected not only by the shear rate and time that the force is applied, but also by many other factors, including the viscometer model, sample temperature, sample preparation technique, and homogeneity of the sample. In this study, in order to make the viscosity of the fluids comparable, the apparent viscosity was recorded and the experiments were all performed under the same conditions unless stated otherwise.

3.1.2 Studies in this chapter

Viscosity is a measure of the flow properties of a liquid; a high viscosity indicates a slow flowing liquid. A frequent reason for the measurement of rheological properties is to characterise and classify fluids and semi-solids. Flow behaviour is responsive to properties such as molecular weight and molecular weight distribution. Viscosity of semi-solids and biological materials produces useful correlations with bioavailability and function (Aulton, 1988). In this project the retention experiments may have been greatly affected by the viscosity of the sodium alginate solutions and this chapter explores this.

The viscosity of alginate solutions is determined by many factors, such as molecular weight or the type of alginate, temperature, solution pH and concentration. As stated by Fuongfuchat et al (1996); mucin synergistically increases the viscosity of solutions when it associates with polymers like alginate or polyacrylate, which is indicative of mucoadhesive potential of alginate. This chapter examines how a range of factors may

affect the viscosity of an alginate solution, particularly with reference to the retention of the alginate solution. Factors including alginate concentration and solution pH were studied.

The rate at which the alginate solution mixed with the water and thus diluted the alginate 2 % (w/v) solution was measured. This process is termed dissolution of the alginate solution within this chapter. The dissolution rate of sodium alginate solutions into distilled water was investigated. In the retention experiments (chapter 2), the alginate was washed off the tissue by water in two different ways. Firstly, the shear force of water flowing over the alginate-tissue surface washed most of the alginate off the tissue surface. Secondly, some of the alginate dissolved from the adhered layer into the flowing water. This study investigated the rate at which solutions of sodium alginate undergo dissolution into distilled water.

3.2 MATERIALS AND APPARATUS

3.2.1 Materials

Sodium alginate powders, H120L, LF120L and LFR5/60 supplied by FMC Biopolymer. Different concentration solutions were prepared according to the experimental needs. The compositions of phosphate buffers were described in detail in Table 2.2. Three pH buffers pH 4, 6, 8 were used. Mucin type II and pepsin (P7012) were purchased from Sigma.

3.2.2 Apparatus

3.2.2.1 Brookfield viscometer DV-I

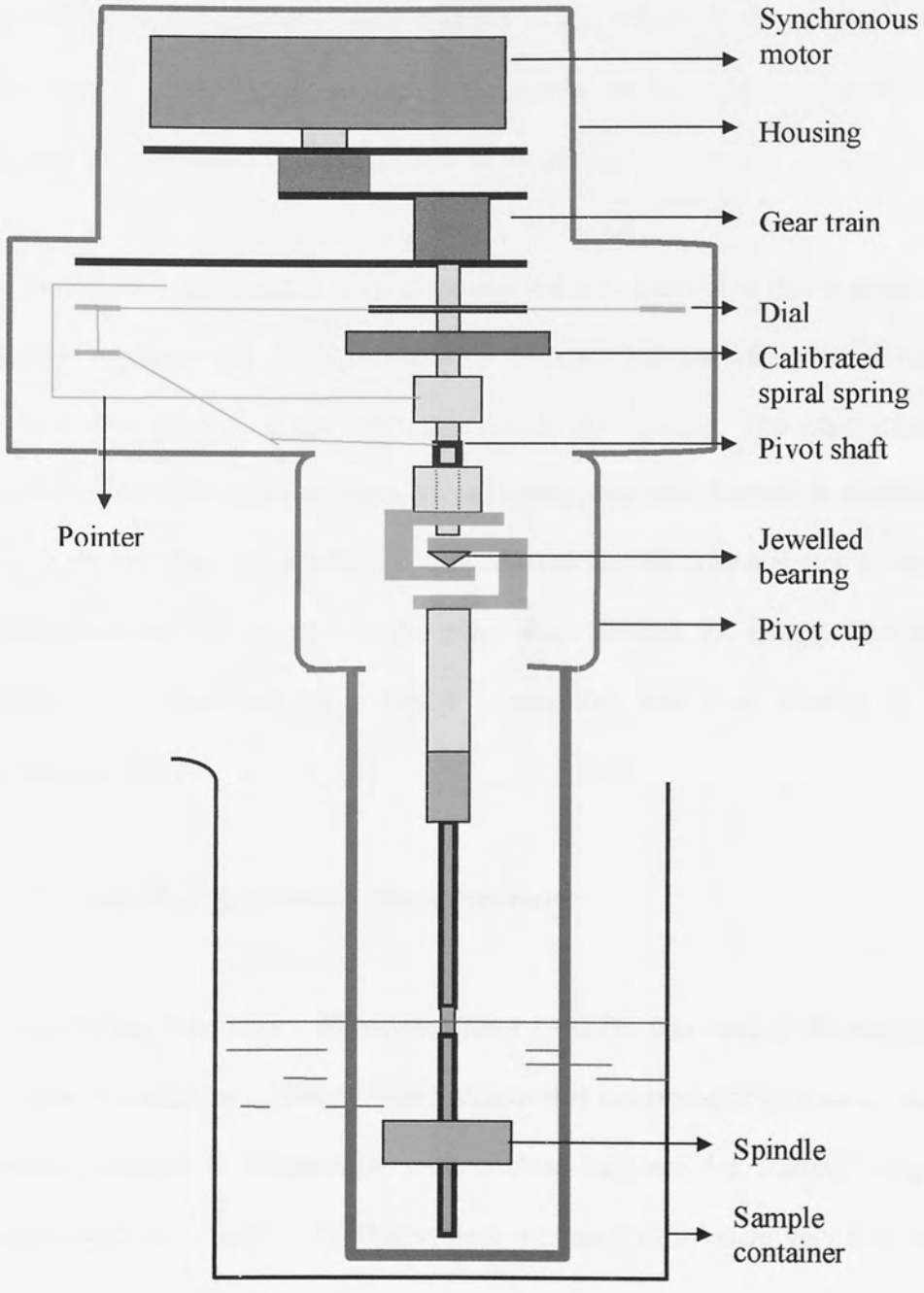


Figure 3.1 Schematic view of the structure of a Brookfield Viscometer

A Brookfield DV-I was used in this study with the guard leg. The working principal of the Brookfield viscometer is to measure the torque required to rotate an immersed element (the spindle) in a fluid. The spindle is driven by a synchronous motor through a calibrated spring. For a given viscosity, the viscous drag, or resistance to flow is proportional to the spindle's rotation speed and is related to the spindle's size and shape. For a given spindle geometry and speed, an increase in viscosity will be indicated by an increase in the deflection of the spring.

The viscometer comprised of several mechanical sub-assemblies that is schematically presented in Figure 3.1. A synchronous drive motor and multiple-speed transmission are located at the top of the instrument inside the housing. The main case of the viscometer is a calibrated beryllium-copper spring, one end of which is attached to the pivot shaft; the other end of which is connected to the dial. The dial was driven by the transmission and, in turn, drives the pivot shaft through the spring. The viscosity measured was displayed on a digital screen that was read directly to gain a measurement.

3.2.2.2 Anton Paar Automated Microviscometer

An Anton Paar Automated Microsviscometer (AMVn) was used in the measurement of viscosity of alginate solutions. The instrument is composed of a compact electronic measuring system in conjunction with a glass capillary for loading samples for measurement (see Figure 3.2). This system was used to measure very low viscosity values from 0.2 – 20 mPa.s with great sensitivity, and was primarily used to measure the dissolution rate of an alginate solution into distilled water in this chapter.

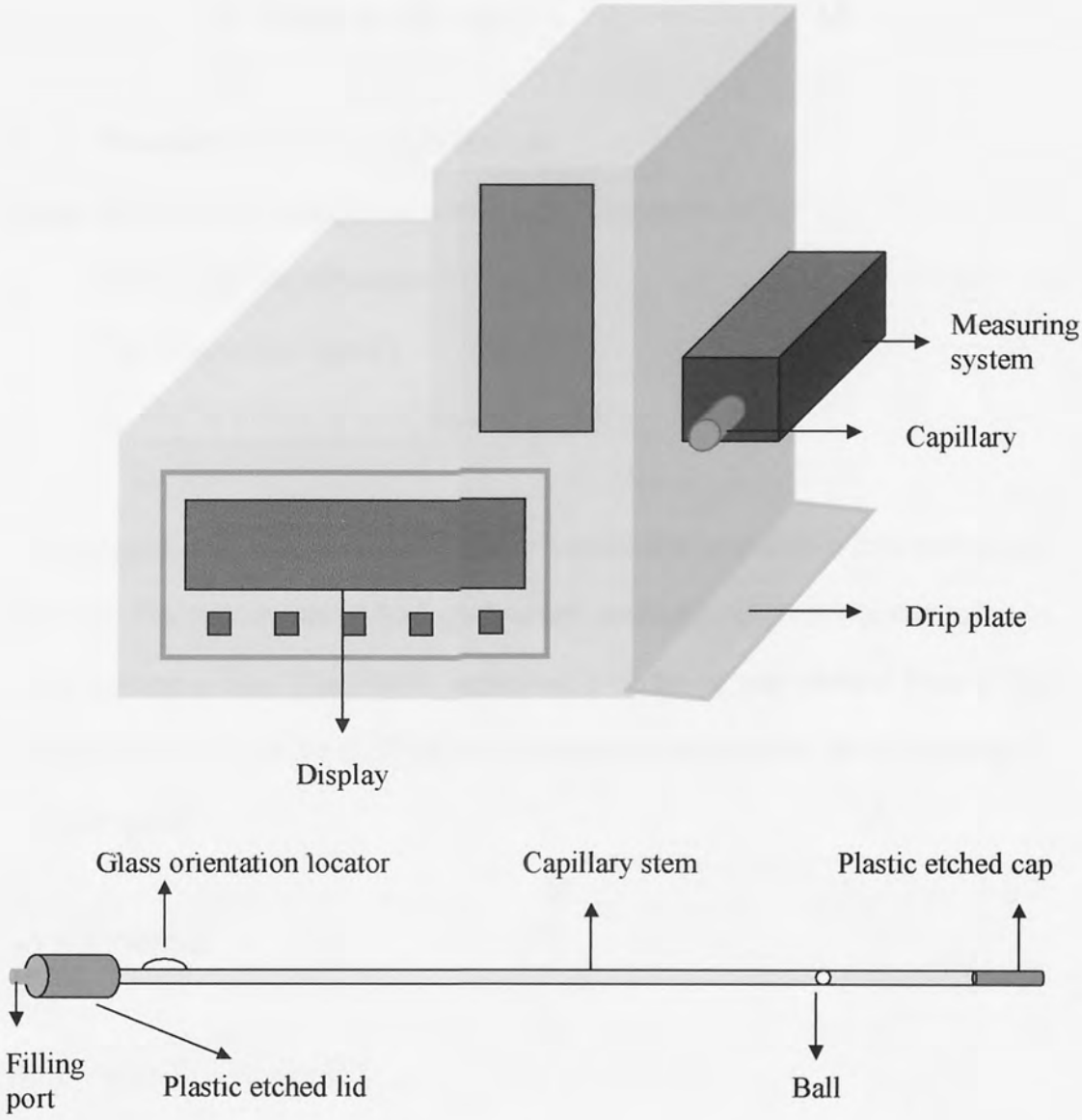


Figure 3.2 Schematic view of Anton Microviscometer and its glass capillary

The basic principal of the instrument is that the capillary is filled with liquid; a steel ball flows through the capillary; the time it takes to cover a defined distance is measured. The dynamic viscosity of the sample is calculated from Formula 3.2 in which $K_1(\alpha)$ is the calibration constant of the measuring system and t_0 is the rolling time.

$$\eta = K_1(\alpha) \bullet t_0 \bullet (\rho_k - \rho_s) \qquad 3.2$$

η Dynamic viscosity of sample (mPa.s)

$K_1(\alpha)$ Calibration constant of the measuring system (mPa.cm^3/g)

t_0 Rolling time for 100mm (s)

ρ_k Ball density (7.85 g/cm^3)

ρ_s Density of sample to be measured (g/cm^3)

Before operation it was ensured that the instrument was placed on a firm surface and balanced. The temperature of the liquid sample inside the capillary was controlled by the measuring system. The AMVn measuring programme was selected from a drop down list; a 70 x 4 was the standard test to measure liquid viscosity via an angle of 70° and four repeats.

3.3 METHODS

3.3.1 Viscosity measurement

Preparation of the solution: a Heidolph rotary mixer was used to make the polymer solutions. The calculated mass of powder was gently poured into the beaker with 100 mL accurately measured water, whilst stirring at 2000 rpm. After stirring for 2 hours there were no visible clumps in the beaker, the beaker of viscous liquid was removed from the mixer and left to equilibrate for 5 minutes.

The guard leg was present with the Brookfield viscometer and spindle 4 was attached to the viscometer. Care was taken not to break the spring of the machine when screwing the spindle on. The viscosity of the liquid was read after a set period of time.

Because of the high viscosity of some liquids a longer time was needed to wait for the liquid to recover the original form after the guard leg and the spindle were inserted.

Sodium alginate liquid is a pseudoplastic fluid, with thixotropic properties. Its viscosity changed with time at a constant shear rate. In order to make the apparent viscosity comparable, all of the readings and experimental conditions were kept the same. All of the experiments were performed at room temperature except when temperature was a study subject. The same viscometer (Brookfield DV-I) and the same spindle (No. 4) were used, the rotation speed was set at 12 rpm. The sample container was a 100 mL standard beaker. During preparation of the alginate liquid, the speed and duration of stirring were all the same at 2000 rpm and 2 hours, respectively, the reading was recorded after the spindle had rotated for five minutes.

In the retention study, the higher the concentration of alginate solution, the greater the observed viscosity and more retention of alginate. That is the reason why this viscosity study was performed. Many factors in gastric refluxate were considered to affect the viscosity of sodium alginate solutions, including mucin, pepsin, and different pH values. Different concentrations of liquid alginate were used in the retention study and that did affect the retention results, the concentration was therefore included in this study. The length of equilibration time of the solution after stirring and the duration of applied shear force were also examined within this study.

3.3.2 Microviscosity

As stated in the introduction of this chapter, there are two ways that the retention procedure washed the alginate from the oesophageal epithelia. One is the shear force

that removes most of the alginates; the other is dissolution of the applied alginate into the flowing media (artificial saliva) caused by the attractive molecular forces between the viscous liquid and the washing media liquid. The hydrophilic carboxylate groups, on the chain units M and G are attracted by the water molecule, this molecular force drags the alginate molecules from the viscous liquid to dissolve into the flowing media. The percentage of the alginate that was removed by dissolution into the washing media was examined in this study.

Table 3.1 The concentrations of alginate used in the Microviscosity study

Alginates	*H120L*	*LF120L*	*LFR5/60*
Concentrations of the alginate(w/v)	*1 %, 2 %, 3 %, 4 %*	*1 %, 2 %, 4 %*	*2 %, 5 %, 10 %*

This study presumed that at very low concentrations the relationship between alginate concentration and viscosity would be linear, and thus a calibration curve was prepared. A series of alginate solutions was prepared. Each alginate was diluted via a series of concentrations listed in Table 3.2. The viscosities of these solutions were measured using Anton Paar automated Micro-Viscometer (AMVn) to produce a calibration curve of viscosity (cp) verses concentration % (w/v).

Table 3.2 Concentrations of aqueous alginate used for setting up calibration curve

Alginate	Concentrations (w/v)
H120L	0.001%, 0.002%, 0.004%, 0.006%, 0.008%, 0.015%
L120L	0.002%, 0.005%, 0.008%, 0.01%, 0.02%
LFR5/60	0.002%, 0.0025%, 0.004%, 0.005%, 0.01%, 0.025%, 0.04%

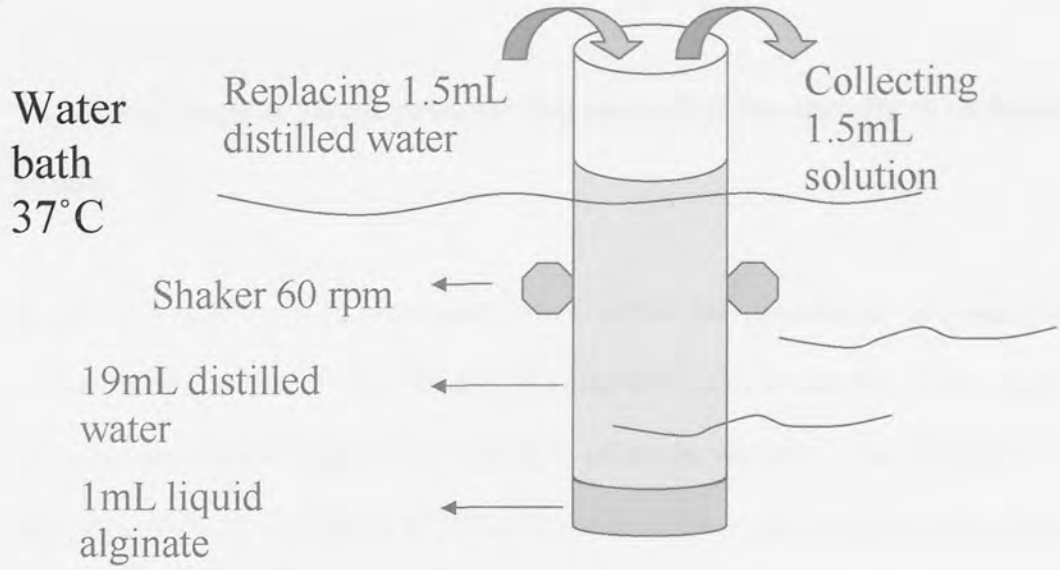


Figure 3.3 Schematic representation of the procedure for the microviscosity study

Experimental procedure to measure the dissolution is shown schematically in Figure 3.3. 1 mL of the alginate solution under test was added to a screw top vial (30 mL volume) that contained 19 mL of distilled water. This alginate was added using a syringe to place the alginate at the base of the vial. The vials were fixed in the shaker within a water bath at physiological temperature 37 °C. The shaking rate was set to 60 rpm. At set time points (1, 5, 10, 15, 20, 25, 30 min) 1.5 mL sample was withdrawn and the same volume of distilled water replaced the sample volume. The viscosity of the samples was measured using the Micro-Viscometer.

3.4 RESULTS AND DISCUSSIONS

3.4.1 Components of gastric refluxate that may affect the viscosity of an alginate solution

Mucin is present both in swallowed saliva within the oesophagus and also from refluxed gastric contents. Thus there is potential for mucin to interact with an applied alginate layer and this interaction is likely to affect the viscosity. The viscosity of an alginate solution (2 % LF120); a 10 % m/v mucin solution and a combination of these solutions (1:1 ratio, total polymer concentration was 2 % alginate and 10 % mucin) was measured within this study. The data and diagram are presented in Table 3.3 and Figure 3.4.

Table 3.3 Apparent viscosity (cp) of the alginate solution LF120L 2 % (w/v) compared with mucin suspension 10 % (w/v) and LF120L 2 % (w/v) mixed with mucin 10 % (w/v), n=4, mean ± s.d., (Brookfield Viscometer; spindle 4, rpm=12)

	Viscosity (cp)		
Time (min)	2 % LF120L	10 % (w/v) mucin	2 % LF120L and 10 % mucin
0	1413 ± 220	68.7 ± 11.7	6490 ± 510
10	1386 ± 147	65.0 ±12.4	6328 ± 500
30	1380 ± 180	63.8 ± 14.8	5970 ± 545
60	1386 ± 113	63.5 ±14.5	5958 ± 625
120	1406 ± 141	62.3 ± 14.1	6270 ± 319
1440	1313 ± 180	62.5 ±7.9	6535 ± 158

Table 3.3 and Figure 3.4 show that the viscosity of LF120L was around 1400 cp and 10 % mucin was 65 cp. But when both of polymer mixed together, the viscosity was up to around 6000 cp, about 400 % higher than sum of both polymer liquid that means

the mucin significantly increased the viscosity of LF120L 2 % (w/v). As stated by Fuongfuchat et al (1996) the synergistic increase in viscosity of a solution with mucin is indicative of mucoadhesive potential. The viscosity had the trend of reducing over time but there was no significant difference in the values at the time points measured.

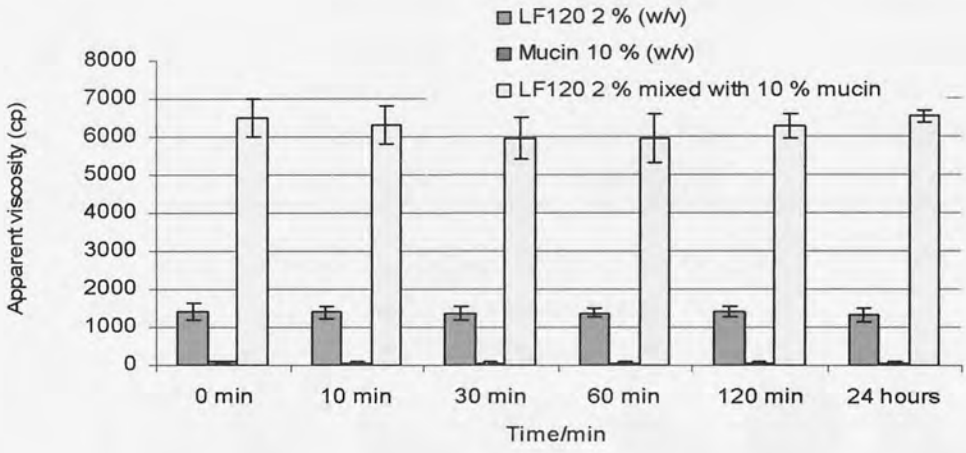


Figure 3.4 Viscosity of the alginate solution LF120L 2 % (w/v) compared with mucin suspension 10 % (w/v) and LF120L 2 % (w/v) mixed with mucin 10 % (w/v) (n=4, mean ± s.d.) (Brookfield Viscometer DV-I; spindle 4, rpm=12)

Pepsin is a damaging component of gastric refluxate and it may have an effect on alginate viscosity, this was measured within this study. The viscosity of an alginate solution alone and in combination with both a 0.05 % w/v and 0.1 % w/v solution of pepsin was measured. The viscosity measurement results are listed in Table 3.4 and Figure 3.5.

Table 3.4 The effect of the addition of pepsin (0.05 % or 0.1 %, w/v) on the viscosity of alginate LF120 2 % (w/v) solutions measured using Brookfield viscometer DV-I; spindle 4, rpm=12, (n=4, mean ± s.d.)

Time (min)	2 % LF120L	2 % LF120L and 0.05 % pepsin	2 % LF120L and 0.1 % pepsin
0	1413 ± 220	1140 ± 227	1240 ± 245
10	1386 ± 147	1166 ± 174	1120 ± 0
30	1380 ± 180	1166 ± 141	1113 ± 23
60	1386 ± 113	1227 ± 133	1146 ± 57
120	1406 ± 141	1413 ± 100	1360 ± 139
1440	1313 ± 180	1267 ± 240	1073 ± 306

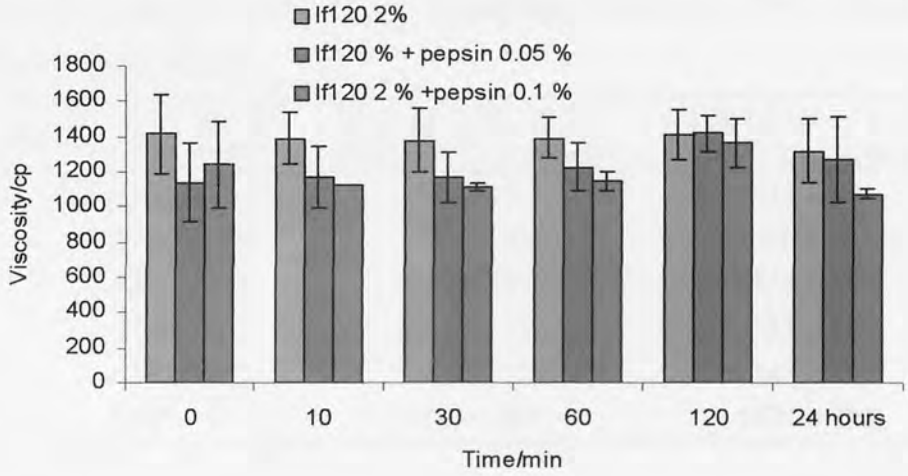


Figure 3.5 The effect of the addition of pepsin (0.05 % or 0.1 %, w/v) on the viscosity of alginate LF120 2 % (w/v) solutions measured using Brookfield viscometer DV-I; spindle 4, rpm=12, (n=4, mean ± s.d.)

The addition of pepsin to the solution of alginate reduced the overall viscosity of the solution, however, this reduction was not statistically significant. Thus the viscosity of alginate in the presence of pepsin is not greatly affected. Pepsin is an enzyme that breaks down proteins and its function in the stomach is to aid in the digestion of food.

However, this study suggests that alginates are not significantly degraded in the presence of pepsin.

A separate study examined the effect on viscosity upon addition of both pepsin and mucin to a solution of alginate. Table 3.5 and Figure 3.6 list the results of alginate (LF120L, 2 % w/v) mixed with 10 % w/v type II mucin and pepsin at concentration of 0.05 % w/v and 0.1 % w/v.

Table 3.5 The effect of addition of both mucin and pepsin to the apparent viscosity of an alginate solution measured on Brookfield Viscometer DV-I (spindle 4, rpm=12), n=5, mean ± s.d.

Time (min)	2 % LF120L and 10 % mucin	2 % LF120L, 10 % mucin, 0.05 % pepsin	2 % LF120L, 10 % mucin, 0.1 % pepsin
0	6490 ± 510	5733 ± 1375	4918 ± 850
10	6328 ± 500	5625 ± 1333	4538 ± 810
30	5970 ± 545	5455 ± 1310	4455 ± 812
60	5958 ± 625	5480 ± 1372	4355 ± 765
120	6270 ± 319	5628 ± 1217	4343 ± 897
1440	6535 ± 158	6220 ± 481	5273 ± 781

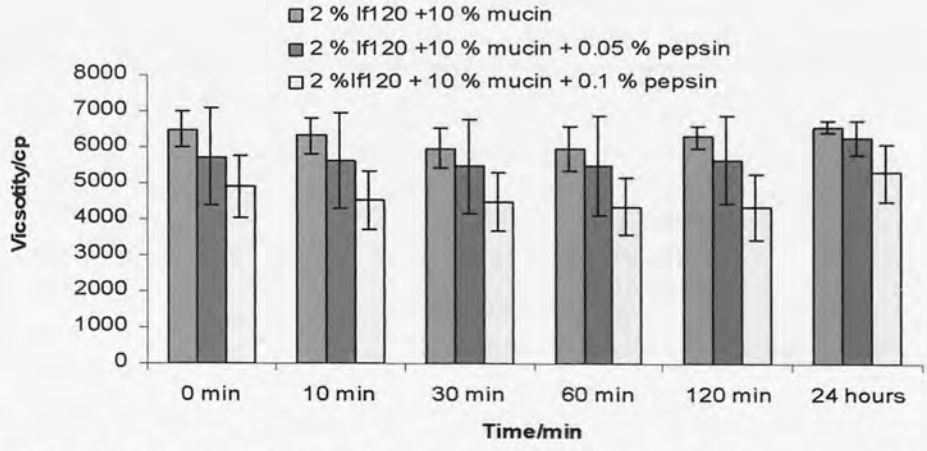


Figure 3.6 The effect of addition of both mucin and pepsin to the apparent viscosity of an alginate solution measured on Brookfield Viscometer DV-I (spindle 4, rpm=12), n=5, mean ± s.d.

Pepsin in gastric contents was reported to degrade the viscosity of polymer solution (Pearson et al, 1986). In Table 3.5 and Figure 3.6, the apparent viscosity values were significantly decreased by addition of pepsin 0.1 % in LF120L 2 % (w/v) solution and solution of LF120L 2 % (w/v) mixed with mucin 10 % (w/v) at most time points. But there was no significant difference noted when pepsin was present at a concentration of 0.05 % w/v. This result suggests that only when pepsin concentrations are 0.1 % w/v or greater, that the viscosity of the alginate-mucin solution is reduced. These results show that mucin - a glycoprotein (Quissell & Tabak, 1989) is susceptible to degradation at high concentrations of pepsin as evident by the reduction in viscosity observed above.

Table 3.6 Apparent viscosity of alginate (LF120L) in different pH buffers over time (n=4, mean ± s.d) measured on Brookfield Viscometer DV-I (spindle 4, rpm=12)

Time (hrs)	2 % LF120L in pH 4 buffer	2 % LF120L in pH 6 buffer	2 % LF120L in pH 8 buffer
2	1247 ± 50	1087 ± 81	807 ± 31
24	960 ± 60	780 ± 35	647 ± 64
48	813 ± 23	733 ± 31	567 ± 58
72	740 ± 72	693 ± 64	540 ± 20
96	553 ± 12	533 ± 31	507 ± 11

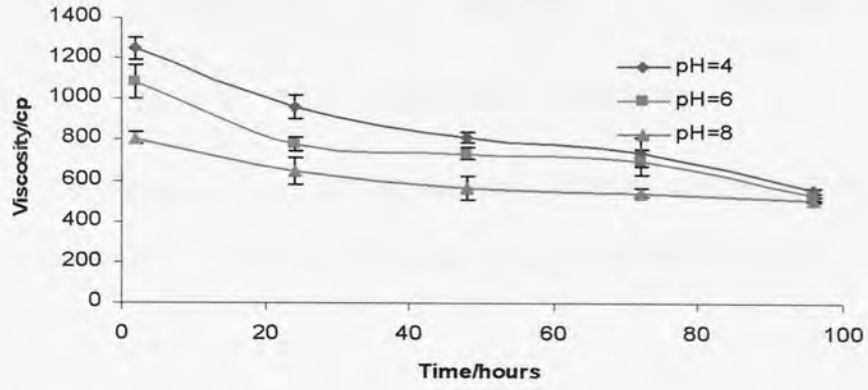


Figure 3.7 Apparent viscosity of alginate (LF120L) in different pH buffers over time (n=4, mean ± s.d) measured on Brookfield Viscometer DV-I (spindle 4, rpm=12)

Table 3.6 is plotted in Figure 3.7 which demonstrates that the apparent viscosity significantly decreased as pH increased at most time points. The explanation for this may be that higher concentration of hydrogen ions can increase the interaction of the alginate molecular chains, as the number of hydrogen bonds linking the chains increase, thus increasing the apparent viscosity. The starting apparent viscosity was significantly higher than that at the later time points, which is likely to be due to the thixotropic nature of the alginate solution.

Table 3.7 Apparent viscosity of alginate (LF120L) at different concentrations (n=4, mean ± s.d) measured on Brookfield Viscometer DV-I (spindle 4, rpm=12)

Concentration of alginate (LF120L)	1 % (w/v)	2 % (w/v)	4 % (w/v)
Apparent viscosity (cp)	20 ± 0	1147 ± 160	143000 ± 1692

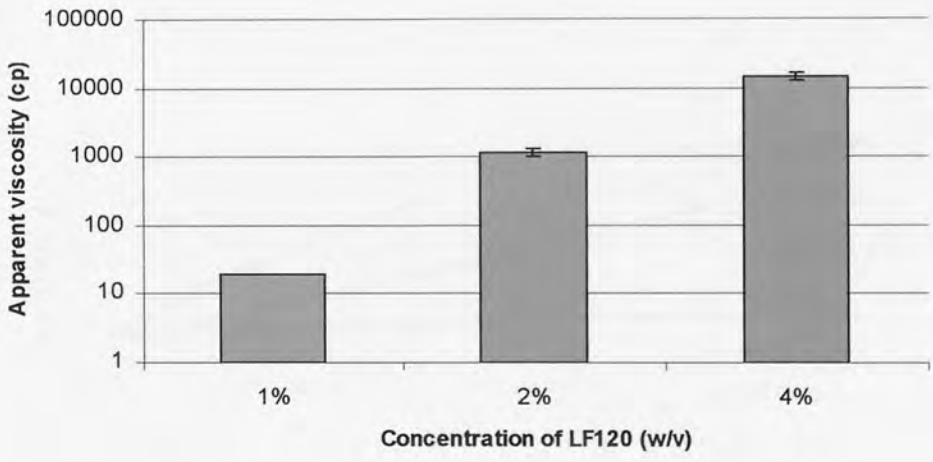


Figure 3.8 Apparent viscosity of alginate LF120L 2 % (w/v) at different concentrations (n=4, mean ± s.d.) measured on Brookfield Viscometer DV-I (spindle 4, rpm=12)

Figure 3.8 and Table 3.7 demonstrate, as expected, that as the concentration of alginate (LF120L) increased the apparent viscosity of the liquid also increased. No linear relationship was found between concentration and viscosity (not that the viscosity if plotted on a log scale).

3.4.2 Microviscosity

Calibration

When the viscosity (or the concentration) of an alginate solution is very small, a linear relationship exists between viscosity and concentration. The calibration of the alginate solutions was performed according to this theory. Figure 3.9 shows the calibration for each of the sodium alginate solutions. The maximum viscosity measured for each alginate was limited to within the linear region.

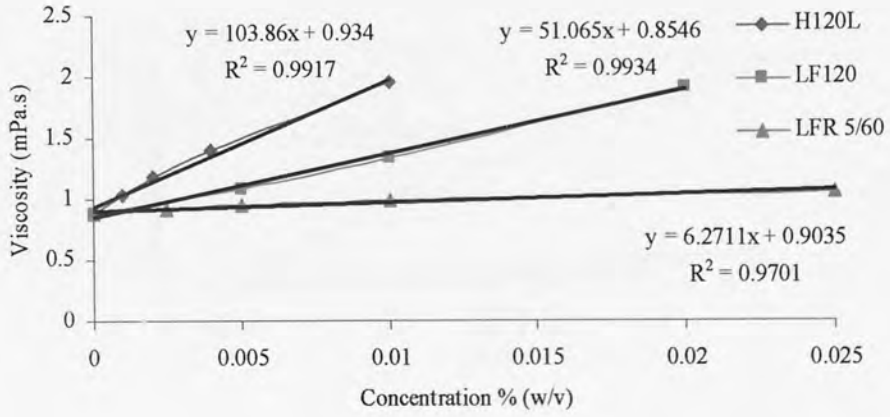
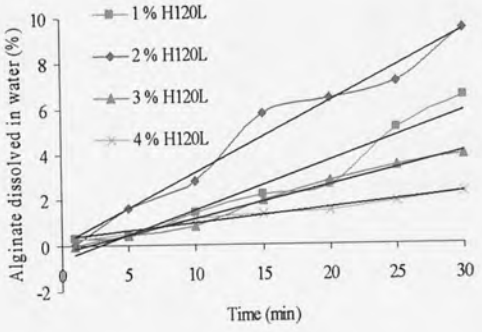


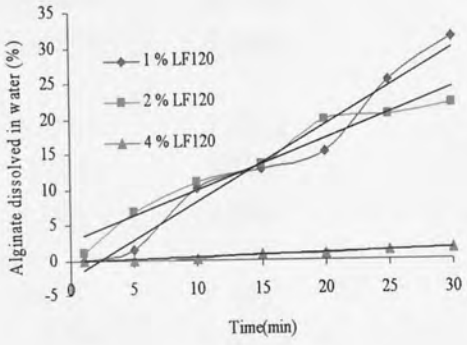
Figure 3.9 Micro-viscometric calibration of the relationship between the viscosity and concentration for three solutions of sodium alginate measured on Anton Micro-Viscometer

Using the calibration equation, the percentage of the alginate dose dissolved in water over time was calculated. The profile of the percent of the dose dissolved over time was drawn and the rate of dissolution was calculated as the gradient of this line. The effect of concentration of alginate solution on the rate of dissolution was examined. The three alginates with different concentrations (H120L 1%, 2%, 3 %, 4 %; LF120L 1 %, 2 %, 4 %; LFR5/60 2 %, 5 %, 10 %) dissolution results are shown in Figure 3.10.

a.



b.



c.

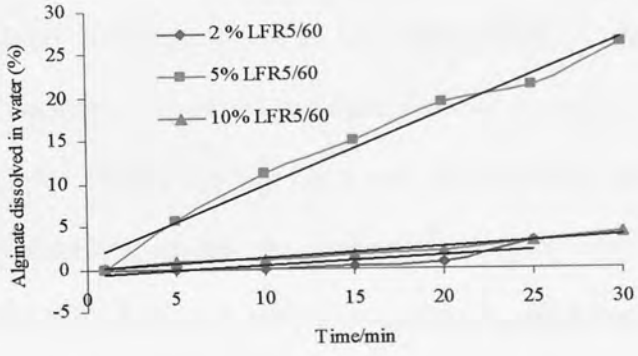


Figure 3.10 The effect of alginate concentrations on dissolution rate for three alginates using Anton Micro-Viscometer (a = H120; b = LF120L and c = LFR5/60)

The dissolution equations are shown in Table 3.8 alongside the dissolution rate for each solution examined.

Table 3.8 The dissolution equations and dissolution rates for liquid alginates with a range of concentrations dissolving in water within 30 minutes

Alginate solution	Dissolution equations	Dissolution rate (%/min)
H120l		
1%	y = 0.2151x - 0.6096	0.2151
2%	y = 0.3127x + 0.03	0.3127
3%	y = 0.1458x - 0.2834	0.1458
4%	y = 0.0662x + 0.2785	0.0662
LF120L		
1%	y = 1.0712x - 2.5205	1.0712
2%	y = 0.7107x + 2.7222	0.7107
4%	y = 0.0561x - 0.226	0.0561
LFR5/60		
2%	y = 0.105x – 0.6612	0.105
5%	y = 0.8514x + 1.1286	0.8514
10%	y = 0.1277x - 0.065	0.1277

The results in Table 3.8 suggest that as the concentration of alginate increases the dissolution rate decreases. There are two exceptions to this rule; 1 % H120L and 2 % LFR5/60. The 2 % LFR5/60 solution has a very low viscosity and it may be that the solution is fully dissolved or that the changes in viscosity were not detected. The unusual results for 1 % w/v H120L may be caused by experimental error.

The method of analysing the data to get the percentage of the alginate dose dissolved in water after 30 minutes shaking in water is described below.

According to the calibration, the concentration of the alginate solution after 30 minutes was determined, this value was converted to the percentage of the alginate dose dissolved in water at 30 minutes as follows:

$Vis_{30min} \rightarrow Con_{30min} \rightarrow \%_{Alg\ dose\ dissolved\ in\ water} = Con_{30min} * 20\ mL / mass_{\ alg\ applied}$.

Vis = viscosity

Con = concentration

e.g. LF120 2 % (w/v) 1mL was dispensed into 19 mL distilled water, after 30 minutes shaking, the solution viscosity was 1.9801 cp, according to the calibration;

$$y = 51.065x + 0.8546$$

The concentration of this solution at 30 minutes, x was calculated;

$$x = (y-0.8546)/51.056 = (1.9801-0.8546)/51.056 = 0.02187\ \%\ w/v$$

The entire dose applied in vial was 2 % * 1mL = 0.02 g

The mass of 2 % alginate dissolved in water at 30 minutes was;

Concentration at 30 minutes * volume = 0.02187 % * 20 = 0.004374 g

So the percentage of alginate LF120 2 % dissolved in water at 30 minutes was,

0.004374/0.02 * 100 % = 21.88 %

A summary calculation result for all of alginates dissolution at 30 minutes is shown in Table 3.9 and Figure 3.11 plots the relationship between the concentrations and the dissolution percentage of alginate.

Table 3.9 The percentage of applied alginate dose that has dissolved into water at 30 minutes

Alginate solution	% Alg dissolved in water
H120L	
1%	6.506
2%	9.485
3%	3.93
4%	2.276
LF120L	
1%	31.25
2%	21.88
4%	1.414
LFR5/60	
2%	29.15
5%	25.54
10%	4.109

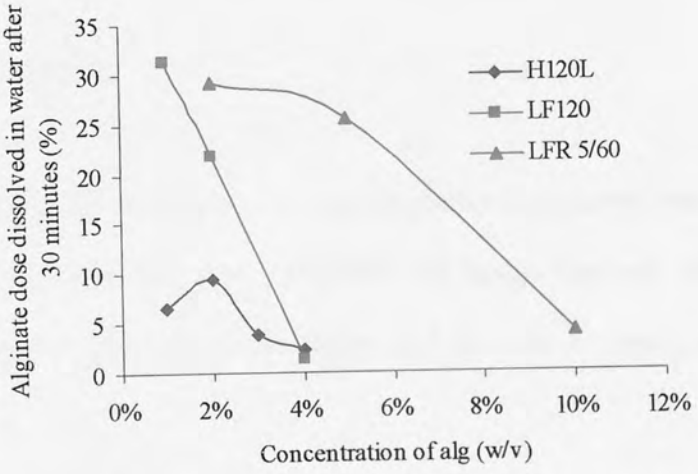


Figure 3.11 A graph to display the relationship of alginate concentration vs. percentage of dissolved applied dose after 30 minutes

Figure 3.11 clearly demonstrates that less dissolution occurred for more concentrated alginates. It is probably due to the larger number of interactions present in the more concentrated solutions thus they are more tightly bound as an alginate "gel" and it will take more energy to disrupt this "gel" and allow dissociation into the surrounding water.

Within the experimental apparatus the surface area for contact between the alginate solution and the water remained constant. Dissolution of the alginate was from this upper surface area into the surrounding water and the degree of dissolution was measured from the alginate concentration within the bulk liquid. When a high concentration of alginate was present the percentage of the total dissolved was lower than a corresponding low concentration although the actual mass of alginate dissolved was greater. The number of alginate molecules present at the interface between the alginate and water is greater for higher concentrations of alginate therefore a greater number of molecules are free to dissolve, yet as a percentage the overall dissolution will be reduced.

3.5 CONCLUSIONS

Viscosity, one of the most important physicochemical properties was used to examine the alginate liquids in this study. The different factors that may affect the viscosity were investigated. From the above results and discussions some conclusions can be made.

As expected 10 % (w/v) mucin greatly increased the viscosity of LF120L 2 % (w/v), which is agreement with the work done by Fuongfuchat et al (1996) demonstrating that mucin mixed with alginate substantially increased the viscosity of the system. When pepsin, 0.1 % (w/v) was added to LF120 2 % (w/v), in combination with 10 % (w/v) mucin, the viscosity was significantly lowered. The viscosity of alginate at lower pH was significantly higher than at high pH. Concentration change of aqueous alginate presented a visible difference in viscosity and the data showed the same results that increasing concentrations of alginate liquid greatly increased the viscosity of these

liquids. Time was examined as a factor that affected the viscosity of an alginate solution. Although there was no significant difference in viscosity between adjacent time points, all viscosities gradually decreased over time. Thixotropy of the alginate solution provides an explanation for this phenomenon.

In the study of microviscosity, the results demonstrated that the higher concentration of alginate liquid or the higher viscosity of the liquid, the less percentage of applied dosage would be dissolved in washing media.

CHAPTER 4 DIFFUSION STUDY

4.1 INTRODUCTION

Reflux symptoms may result from an imbalance between an excess exposure to acid and pepsin and inadequate defence mechanisms. As stated in chapter 1 (Introduction) the stomach and duodenum, have both a well-defined mucus layer that provides a protective coating against the acidic environment and goblet cells secreting bicarbonate to neutralise acid. Mucin molecules undergo a sol-gel transition at low pH due to cross-linking of the molecules through hydrophobic interactions (Cao et al. 1999). This gel is resistant to the back-diffusion of secreted acid and maintains a pH gradient from pH 2 in lumen to pH 7 at the apical cell surface (Khanvilkar et al. 2001). The thickness of the mucus layer in the human stomach has been reported to be 576 μm by Bickel and Kaufman (1981) whereas Allen (1981) reported a mean thickness of 192 μm for a continuous mucus layer. However, the pre-epithelial defences within the oesophagus comprise an unstirred water layer that has a thickness in the range of 30-95 μm (Attwood, 1994; Sarosiek et al, 1993). This layer can only maintain a pH gradient of approximately 1 pH unit.

Previous *in-vitro* work has demonstrated that solutions of sodium alginate adhere to porcine oesophageal mucosal for up to sixty minutes (Batchelor et al, 2002). This study investigates whether these adhesive alginate oesophageal bandages assist the pre-epithelial defences against both acid and pepsin. The diffusion of acid and pepsin

in an acidified solution through alginate formulations was investigated. A reduction in the rate of acid reaching the epithelial cell layer indicates an enhanced pre-epithelial defence and may reduce the requirement for systemically administered therapies for GORD.

4.1.1 Theory of acid diffusion

The permeation coefficient of the diffusion barrier (P) and the thickness of the barrier (h) may be determined by the permeation of hydrogen ions according to Fick's first law,

$$P = (J*h)/C \qquad \textbf{Equation 4.1}$$

Where J = the flux of the diffusate through the layer and C = the initial concentration of the drug in the donor. J is defined as:

$$J = M/At \qquad \textbf{Equation 4.2}$$

Where M = the mass of the diffusate present in the receptor of a diffusion cell (described in 4.3.1) at time t, A is the area available for diffusion. In a graph of mass per area that has diffused against time the flux can be calculated as the gradient of the line, which was used as J, the flux of the diffusate, which also was as the diffusion rate as stated in this study.

Previous work performed by Williams and Turnbery (1980) measured the permeation coefficient of porcine gastric mucus to be 1.75×10^{-5} cm^2/sec compared to 6.65×10^{-5} cm^2/sec for a control, a reduction to 26 % of the control value. Slomiany et al. (1985)

measured the permeation coefficient of mucus and reported a value of 6.51 x 10^{-6} cm^2/sec compared to the control 65.60 x 10^{-6} cm^2/sec. The incorporation of sucralfate into gastric mucin further reduced the permeation coefficient providing evidence that sucralfate strengthens mucus gel and aids in the retardation of acid diffusion. Both the above studies demonstrated that the presence of a mucus layer leads to a significant reduction in hydrogen ion diffusion compared to a control. In this study the presence of alginate layer, to act in a similar way to mucus and retard hydrogen ion diffusion was examined.

4.2 MATERIALS

4.2.1 Sodium alginates

Alginate chains are built as randomised block copolymers from two monomeric sugar units, guluronic (G) and manuronic (M) acid. The chemical and physical properties of alginates were described in detail in 2.2.1.2. Three sodium alginates were investigated in this study; their properties, including the fraction of G units (F_G) and concentrations are listed in Table 4.1.

Table 4.1 The properties of the sodium alginates used in the study

Alginate	Molecular Weight (Da)	F_G	Conc^n investigated
Protanal H120L	416000	0.46	1,2,3 % w/v
Protanal LF120	240000	0.44	1,2,3,4 % w/v
Protanal LFR5/60	40000	0.64	2,5,10 % w/v

The alginate solutions were prepared by slow addition of the measured mass of alginate powder to the designated volume of distilled water under vigorous stirring.

4.2.2 Gaviscon® products

Alginate based liquids are used in the therapy of GORD as they react with acid within the stomach to form a raft that floats on the gastric contents. This raft acts as a physical barrier to reflux and if reflux does occur it reduces the contact of the gastric contents with the oesophageal epithelium. In addition, alginates may adhere to the oesophageal epithelium forming an oesophageal bandage to further protect the oesophagus from damage.

Alginates are active ingredients of several Gaviscon®-type commercial formulations that were investigated in this study; Gaviscon Liquid® and Gaviscon Advance® were supplied by Reckitt Benckiser Healthcare, UK, and Gaviscon Mikstur®, Ferring Pharmaceuticals A/S was purchased from a Norwegian pharmacy.

4.2.3 Hydrochloric acid and pepsin

0.1M and 0.01M hydrochloric acid was prepared by dilution of a 5M solution supplied by Sigma, UK. Porcine gastric pepsin (Sigma, 7012), purchased from Sigma, was prepared as a 0.3 % w/v solution in 0.01M hydrochloric acid. Solid pepsin was stored in the freezer at - 20°C.

4.2.4 Membranes

Dialysis membrane (Fisher Science Ltd.) was used in the acid diffusion study. The molecular weight cut off (MWCO) of the dialysis membrane was 12-14 K Da. Yet the molecular weight of the pepsin A (Sigma, UK) was 35 KDa. Therefore glass microfibre (GF/B) filter paper (Whatman) with diameter 2.5 cm was used in the pepsin diffusion study in place of dialysis membrane.

4.3 METHODS

4.3.1 Diffusion cells

There are several types of diffusion cells. Basic variations include orientation (horizontal and vertical) and receptor compartments: static or flow-flow-through (Bronaugh and Stewart 1985). *Coldman et al. (1969)* designed the first vertical cell and other workers modified this design later (Gummer et al., 1987; Chien and Valia, 1989; Chowhan and Prichard, 1978)

The vertical cell illustrated in Figure 4.1 is comprised of two chambers, the top donor holds the solution under investigation and the bottom receptor contains the receiving solution that can be a buffer solution or just water. A sampling port at the side of the receptor was used for taking samples for analysis. A magnetic stirring bar on the bottom of the receptor continuously agitated the receptor solution. A membrane was mounted horizontally between the two chambers. This type of orientation of the membrane means that the donor chamber was exposed to ambient temperature and humidity, while the lower receptor chamber was maintained at physiological

temperature via a circulating water jacket. In this study the vertical diffusion cell (Franz-cell) was used to investigate the diffusion of hydrochloric acid and acidic pepsin through an alginate layer.

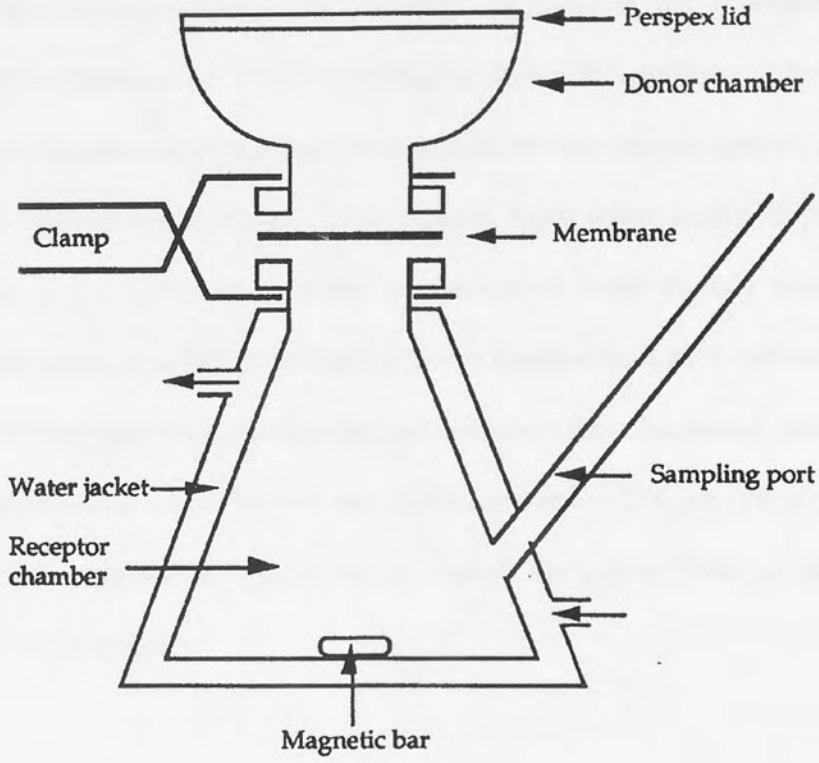


Figure 4.1 Schematic representation of the vertical diffusion cell

4.3.1.2 pH-meter

A BDH Gelplas PH probe attached to a Sartorius pH meter was inserted to the sampling port to measure the solution pH value over time in the receiver of the diffusion cell.

4.3.1.3 Ultraviolet-Visible spectrometer

UV Unicam Helios β spectrophotometer was used in the study of pepsin diffusion to measure the concentration of the pepsin dissolved in hydrochloric acid (pH 2). The theory of the Ultraviolet-Visible Spectrometer to measure the concentration of solutions follows Beer's Law $A = \varepsilon bC$, in which ε, molar absorptivity and b = sample path length which are constant for each fixed sample (In this study it referred to pepsin dissolved in hydrochloric acid). So A, absorbance has a linear relationship with C, concentration of the measured solution. In addition, in order to only measure the absorbance of pepsin in solution, the blank solution (hydrochloric acid solution 0.01 M) was placed in the light path to zero the spectrometer. The absorbance peak of the acidified pepsin solution (0.1 % w/v) was determined at λ = 276 nm (Figure 4.2). At this wavelength a calibration was set up to quantify the concentration of the pepsin dissolved in acid solution.

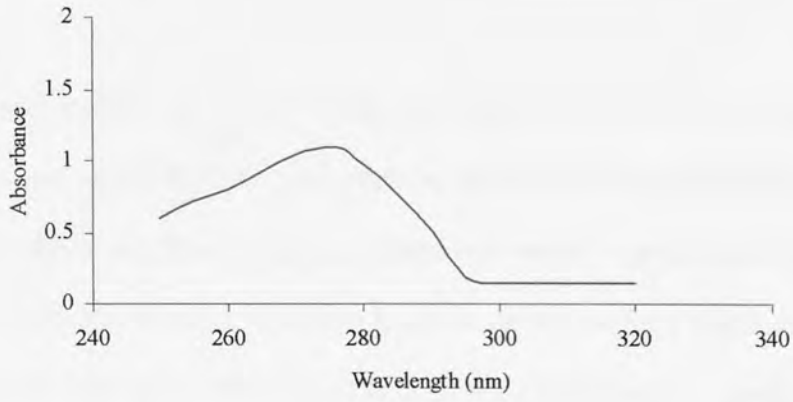


Figure 4.2 UV scan of acidified pepsin solution (0.1 % w/v), peak wavelength was at 276 nm

4.3.2 Experimental methods

4.3.2.1 Acid diffusion studies

In Chapter 2 the retention study confirmed that aqueous alginate applied on oesophageal mucosa and washed by water was retained on the oesophagus for up to 30 minutes. This study examines the reduction of acid diffusion caused by the presence of an alginate layer and postulates that such an adhesive layer on the oesophagus would limit damage caused by gastric reflux

The acid diffusion studies investigated the acid diffusion rate from the upper donor chamber to the lower receptor chamber. The motion of acid was detected directly via a BDH Gelplas pH probe coupled to a calibrated Sartorius pH meter. The diameter of the porthole between the two chambers was 17 mm; the volume of alginate applied was 0.1 mL unless stated otherwise. The area over which diffusion occurred was 227 mm^2.

30mL distilled water was dispensed into the receptor chamber and a magnetic stirring bar was placed on the bottom of the receptor. Hydrated dialysis membrane covered the bottom of the donor chamber and the edge was sealed using Parafilm® to prevent leaking. The donor chamber was then mounted on the receptor chamber and a clamp held the two chambers together. A set volume of aqueous sodium alginate was applied on the membrane surface in the donor chamber and left for a few minutes until the viscous alginate was distributed evenly on the membrane. A pH electrode (BDH Gelplas) was inserted into the solution through the sampling port to measure the pH over time. 25 mL 0.1 M hydrochloric acid solution was gently poured into the donor

chamber taking care to minimise disruption to the alginate layer on the membrane. The timer began once the acid solution had been dispensed. The pH value was recorded at set time points; 20 sec, 40 sec, 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 9 min, 12 min, 15 min, 18 min, 21 min, 24 min, 27 min, 30 min. A control experiment was performed when no aqueous alginate layer was applied on the membrane. All experiments were performed at room temperature.

There are several factors that affect hydrogen ion diffusion including; different types of sodium alginates (at the same concentration the viscosity can differ), different depths of the viscous solutions on the membrane; different concentrations of the same aqueous alginates. So, according to these factors that may affect acid diffusion the studies were performed in different parts; the effect of different aqueous alginates; the effect of different depths of the aqueous alginates; the effect of different concentrations of aqueous alginates; the effect of some commercial formulations.

4.3.2.1.1 The effect of different aqueous alginates

The ability of different alginate solutions H120L 2 % (w/v), LF120 2 % (w/v), LFR5/60 2 % (w/v) to limit acid diffusion was investigated in this study. 0.1 mL of each sample was dispersed on the membrane.

4.3.2.1.2 The effect of different depths of the aqueous alginates

In the retention experiments, most of the alginate layer applied on the oesophageal surface was washed off by distilled water and this action changed the depth of the alginate solution retained on the tissue. This study examines how the depth of the

alginate layer affects acid diffusion. The volumes of the aqueous alginate applied on the membrane were 0.2 mL, 0.1 mL and 0.05 mL. Because the diameter of the donor chamber bottom was fixed at 17 mm, when the applied volume of the aqueous alginate was known, the depth of the solution was fixed, calculated through the formula,

$$D = V/\pi r^2$$

D was the depth of the aqueous alginate layer on the membrane; V was the volume of the applied aqueous alginate; r was the radius of the bottom of the donor chamber here the value was 8.5 mm. Table 4.2 shows the relationship between the depths and the volume of the alginate solutions applied on the tissue.

Table 4.2 Volumes of the alginate vs. depth of the layer on the membrane

The volume of alginates applied (mL)	Depth of the layer on tissue (mm)
0.2	0.88
0.1	0.44
0.05	0.22

The liquid alginates used in this study were three sodium alginates; H120L, LF120, LFR5/60 at concentrations of 2 % (w/v).

4.3.2.1.3 The effect of different concentrations of the aqueous alginates

Viscosity of sodium alginate is proportional to concentration, however as noted in Chapter 3 this relationship is not linear. The effect of alginate concentration on acid

diffusion was measured. The alginates and their concentrations used in this study are listed on table 4.3.

Table 4.3 The concentrations of the alginate used in this study (x indicated the alginates used)

Concentration % (w/v)	H120L	LF120	LFR5/60
1 %	x	x	
2 %	x	x	x
3 %	x	x	
4 %		x	
5 %			x
10 %			x

The same volume 0.1mL (or the depth is 0.44 mm) of each liquid was applied on the membrane as a barrier to reduce acid diffusion. Because of its low viscosity, alginate LFR5/60 was very easily disrupted when acid solution was dispersed into donor chamber. To resolve this problem, after the liquids containing alginate were applied to the membrane, a few drops of acid were dripped onto the alginate layer to make an alginic acid gel. This acidic gel was better able to resist disruption from the application of the donor solution.

4.3.2.1.4 The effect of some commercial formulations (Gaviscon® products)

Alginate based liquids used in the treatment of GORD were examined in this study. Three commercial alginate-based products were tested to measure their ability to reduce the diffusion of acid; Gaviscon Liquid® Gaviscon Advance® and Gaviscon Mikstur®, Ferring Pharmaceuticals A/S 0.1 mL with depth 0.44 mm of each Gaviscon® was applied on the membrane.

Alginate-based products contain sodium alginate as an active ingredient as well as antacid chemicals to neutralise the H^+ ions, and other ingredients including flavouring and some thickeners. Different alginate-based products have different percentages of alginates and antacid chemicals. Table 4.4 shows the ingredients in the different alginate-based products.

Table 4.4 Ingredients in Gaviscon® products

	Gaviscon Liquid®	Gaviscon Advance®	Gaviscon Mikstur®
LFR5/60	5 % (m/v)	10 % (m/v)	5.5 % (m/v)
Antacid	$CaCO_3$;160mg $NaHCO_3$; 267mg NaOH; 26.7mg	$CaCO_3$; 200mg $KHCO_3$; 200mg NaOH; 7.22mg	$CaCO_3$; 150mg $NaHCO_3$; 170mg $Al(OH)_3$; 1000mg
Thickener	Carbopol 974; 65mg	Carbopol 974; 40mg	Xanthan; 20mg
Other additives			

In this study, the capability of different active ingredients including alginate; antacid chemicals and thickeners in reducing acid diffusion was compared. Table 4.5 lists the formulations tested.

Table 4.5 Comparison factors within three Gaviscon® products (control; dialysis membrane only)

Gaviscon Liquid®	**Gaviscon Advance®**	**Gaviscon Mikstur®**
Control	Control	Control
LFR5/60 5 % (w/v)	LFR5/60 10 % (w/v)	LFR5/60 5.5% (w/v)
LFR + Carbopol	LFR + Carbopol	LFR + Xanthan
LFR + antacids	LFR + antacids	LFR + antacids
LFR+antacids+ Carbopol	LFR+antacids+ Carbopol	LFR+antacids+ Xanthan
Commercial product	Commercial product	Commercial product

4.3.2.2 Pepsin diffusion studies

Pepsin is a component in gastric reflux contributing to damage of the oesophageal epithelium (Tobey et al, 2001). So reducing the contact of pepsin with the oesophageal epithelium is as important as reducing the acid contact in protecting the oesophagus from damage by gastric contents reflux. In this study 0.3 g pepsin was dissolved in 100mL hydrochloric acid solution (0.01 M or pH 2) to make the concentration of the pepsin solution 0.3 % (w/v).

Hydrochloric acid 30 mL (0.01 M) was added to the receptor chamber of a Franz cell and a magnetic stirring bar was put on the bottom of the chamber. Filter paper was mounted on the mouth of the receptor chamber, onto which the donor chamber was mounted. The edge of the junction of the two chambers was sealed with Parafilm® to prevent leaking. A set volume of formulation was dispensed onto the membrane - the filter paper, then 20 mL acidified pepsin solution (0.3 % w/v) was gently poured into the donor without disturbing the bioadhesive layer on the membrane. The timer started once the acidified pepsin solution had been administered. A syringe was used to collect samples (1.5 mL) from the sample port at set times; 5 min, 10 min, 15 min, 20 min, 30 min. Once a sample had been collected it was replaced using the same volume of acid solution (0.01 M). The absorbance of the samples were recorded and converted into the concentration of the pepsin solution according to the calibration shown in Figure 4.10. Each experiment was repeated at least five times.

4.3.2.2.1 Factors affecting pepsin diffusion

Three factors that may affect the pepsin diffusion were studied, (A). The types of alginates, including H120L, LF120, LFR5/60 at 2 % (w/v), depth = 0.44 mm; (B). The concentrations of alginates LF120 1 %, 2 %, 3 %, 4 % (w/v), depth = 0.44 mm; (C). The depths of alginates. LF120 2 % (w/v) at depth = 0.22 mm, 0.44 mm, 0.88 mm.

4.3.2.3 Evaluation of the experimental design

There were some factors in the experimental process that might affect the results. Firstly the viscosity of the alginate solution used determined how quickly a homogenous layer that covered the membrane was formed. H120L had a much higher viscosity compared to an equivalent concentration of LFR5/60 and thus H120L took longer for an even layer to form. Secondly, when the spreading liquid touched the glass wall of the donor chamber, capillary action attracted the liquid onto the glass wall and resulted the surrounding layer being thicker than the central part, which made the depth of the layer on the membrane thinner than the calculated value. Thirdly, due to the numbers of experiments performed, the acid used required manufacture of more than one batch thus batch to batch variation in the acid affected the diffusion results. It should be noted that a small change in pH is equivalent to a large change in concentration as pH is measured on a log scale.

4.4 RESULTS AND DISCUSSIONS

4.4.1 Acid diffusion studies

4.4.1.1 The effect of different aqueous alginates

As described in 4.3.2.1.1, three aqueous alginates were used at the same concentration 2 % (w/v) and the same volume 0.1 mL (depth = 0.44 mm) dispersed on the membrane. The pH values recorded at time points were transferred to H^+ concentration (mMol/L, expressed as mM) and plotted against time. The diffusion rate was calculated as the gradient of the line of concentration versus time for each alginate, so the units of acid diffusion were mM/min. To ensure all results were comparable the diffusion rates were compared to a control value that was set at 100 % and the data is expressed as percentages of this value. The lower the percentage value the better the alginate was at reducing the diffusion rate.

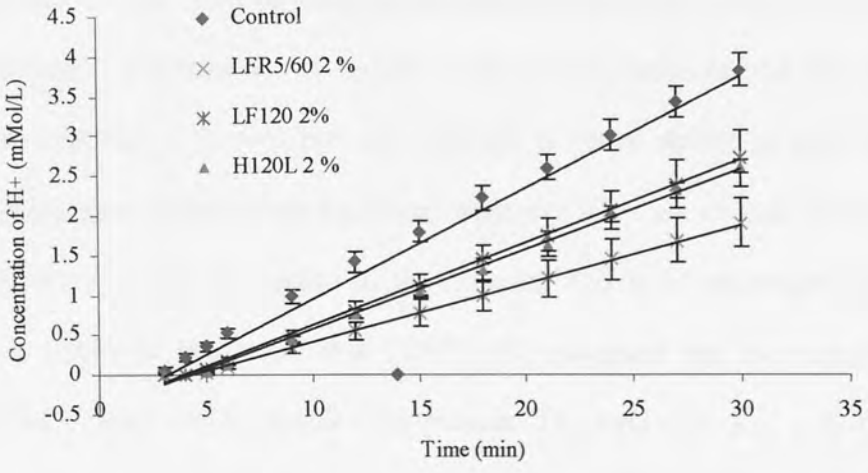


Figure 4.3 Acid diffusion through alginate layer of H120L, LF120, LFR5/60 (2 %, w/v) over time at depth= 0.44 mm (mean ± s.d., n= 4)

The control data was significantly different to the data observed for the three alginates tested. H120 and LF120 showed similar diffusion profiles although LFR5/60 showed reduced diffusion compared to the other alginates.

Table 4.6 Acid diffusion rate in different alginates and the percentage reduction in rate compared with the control (mean ± s.d., n=4)

Hydrochloric acid diffusion rate (mM/min)	% reduction in acid diffusion
Control	0.1374±0.0066	100 ± 4.82
LF120 2 % (w/v)	0.0982±0.0097	71.68±7.08
H120L 2 % (w/v)	0.0994±0.004	72.34±2.92
LFR5/60 2 % (w/v)	0.0821±0.0121	62.47±8.83

Table 4.6 demonstrated that both LF120 and H120L have similar effects in reducing acid diffusion rate at 71.68 ± 7.08 % and 72.34 ± 2.92 % of the control respectively; LFR5/60 had the best effect on reducing the acid diffusion with a rate of 62.47 ± 8.83 % of the control, but there was no significant difference compared with the other two alginates. LFR5/60 2 % w/v not only showed a better ability in acid diffusion reduction but also demonstrated the lowest viscosity. The rate of acid diffusion was inversely related to the G fraction of the alginate. This is in agreement with work previously published by Draget et al (1997) who suggested that the strength of an alginate gel is improved by the G units present. The rigid nature of poly-G blocks within alginate chains means that the exposed acid groups will readily interact with hydroxyl groups leading to the formation of organised hydrogen bonds between two poly G blocks. This association is strong and provides a solid structure for a three

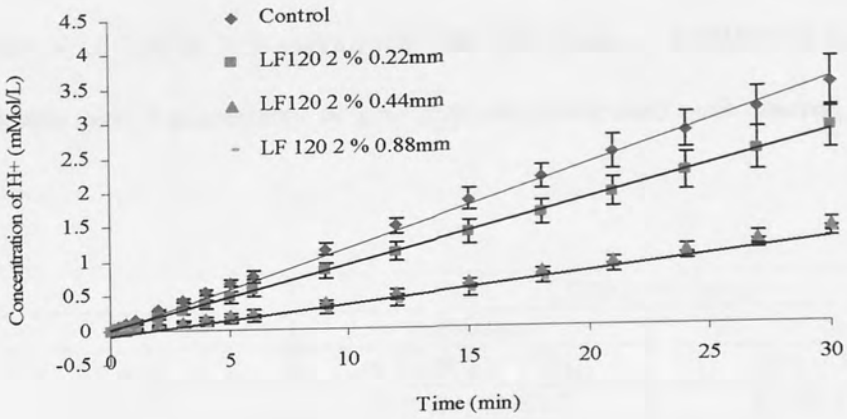
dimensional gel. The number of poly-G-poly-G interactions determines the gel's solid nature and controls the mesh size within the gel, a greater number of associations leads to a smaller mesh size (Draget et al. 1997).

4.4.1.2 The effect of different depths of aqueous alginates

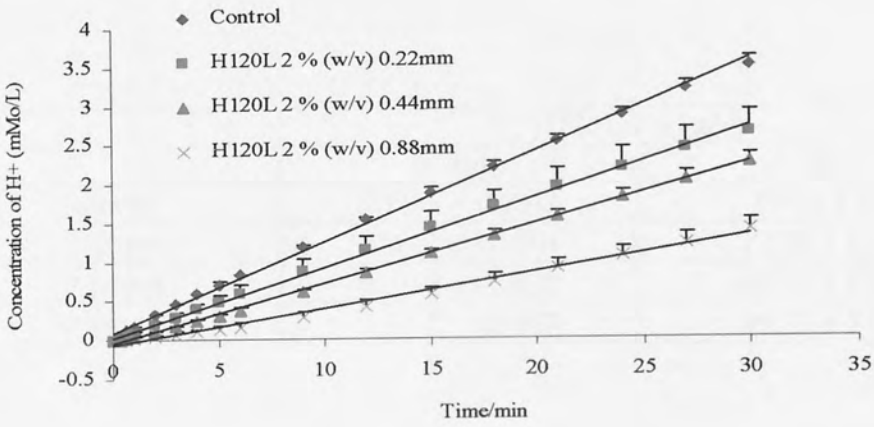
In this experiment the effect of the depth of the alginate gel layer on acid diffusion was investigated. Three alginates liquids with depths (0.22 mm, 0.44 mm, 0.88 mm) were used. Figure 4.4, shows the experimental results.

The diffusion rate was calculated as the linear gradient of the concentration change over time for each alginate (Fig. 4.4) as stated as before. The acid diffusion of the three alginates at 3 depths was calculated and listed in table 4.7.

a.



b.



c

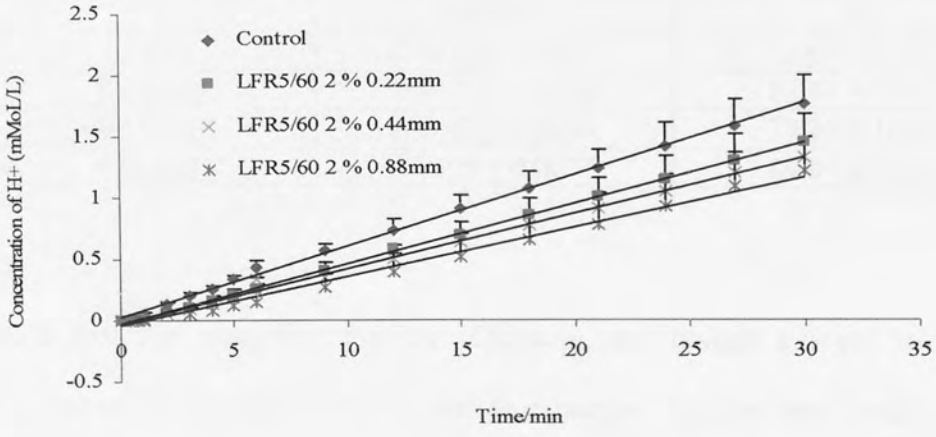


Figure 4.4 Acid diffusion through alginate layers with different depths (0.22mm, 0.44mm, 0.88mm); a. LF120 2 % (w/v); b. H120L 2 % (w/v); c. LFR5/60 2 % (w/v) (n=4, mean ± s.d.)

Table 4.7 Comparison of three alginates with different depths reducing the acid diffusion; a. LF120 2 % (w/v), b. H120L 2 % (w/v), c. LFR5/60 2 % (w/v) (* acid diffusion rate, + percentage of acid diffusion compared with control, n=4, mean ± s.d.)

a

	LF120 2% (w/v)	
	*mM/min	+%
control	0.1139 ± 0.0116	100 ± 10.18
0.22mm	0.0919 ± 0.0100	80.68 ± 8.78
0.44mm	0.0787 ± 0.0030	69.10 ± 2.63
0.88mm	0.0429 ± 0.0026	37.66 ± 2.28

b

	H120L 2% (w/v)	
	*mM/min	+%
control	0.1153 ± 0.0033	100 ± 2.86
0.22mm	0.0891 ± 0.0094	77.28 ± 8.15
0.44mm	0.0762 ± 0.0039	66.09 ± 3.38
0.88mm	0.0458 ± 0.0047	39.72 ± 4.08

c

	LFR5/60 2% (w/v)	
	*mM/min	+%
control	0.0579 ± 0.0083	100 ± 14.30
0.22mm	0.0485 ± 0.0078	83.67 ± 13.50
0.44mm	0.0447 ± 0.0063	77.20 ± 10.88
0.88mm	0.0405 ± 0.0077	69.89 ± 13.30

Fick's first law suggested that the diffusion rate through a layer is inversely proportional to thickness of layer, thus as expected a deeper layer leads to slower diffusion. Due to the increasing path-length of ions getting through, acid diffusion was retarded by thicker depth of alginate. Compared with the control, the acid diffusion rate decreased as bioadhesive layer depth increased. H120L and LF120 at 2 % (w/v)

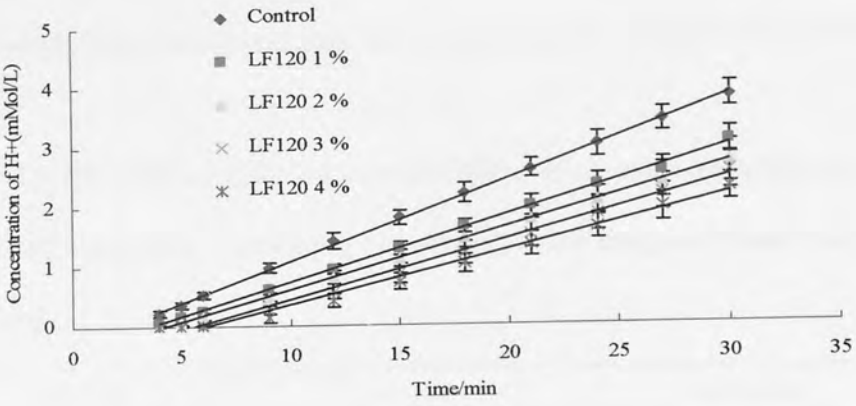
and same depths showed similarities in reducing acid diffusion. The percentage reduction in acid diffusion of the H120L and LF120 gel layers were about 23 % and 20 % at depth 0.22 mm, 34 % and 31 % at depth 0.44 mm, 61 % and 63 % at depth 0.88mm, respectively. The increasing depths significantly increased the reduction in acid diffusion (ANOVA, $P < 0.05$). LFR5/60 2 % (w/v) had a lesser effect than the other two alginates in reducing acid diffusion. At depth 0.22 mm about 17 % reduction, at depth 0.44 mm about 23 % reduction and at depth about 0.88 mm 30 % reduction in acid diffusion were observed. There was no significant difference in the reduction of acid diffusion according to the depth of LFR5/60 applied (ANOVA, $P > 0.05$). This contradicts directly with the results in the previous section where LFR5/60 had the greatest effect, which may be due to the low viscosity of LFR5/60 2 %, when it was applied on the membrane, and "fixed" with acid it was still very susceptible to disruption when the donor solution was poured, the greater the volume, the greater the results were affected, which leads to different results from the previous section. Turner et al. (1985) compared the rate of hydrogen ion diffusion through pig gastric mucus layer of 1 mm thickness and a control saline solution and noted that the presence of mucin reduced the diffusion rate of H^+ to approximately 20 % of the control. Slomiany et al (1985) stated that a 1 mm thickness mucus layer reduced the hydrogen ions diffusion to 10 times lower than a control value. This study indicated that alginate, like mucus, can retard acid diffusion up to 60 % with a depth of 0.88 mm.

4.4.1.3 The effect of different concentrations of aqueous alginates

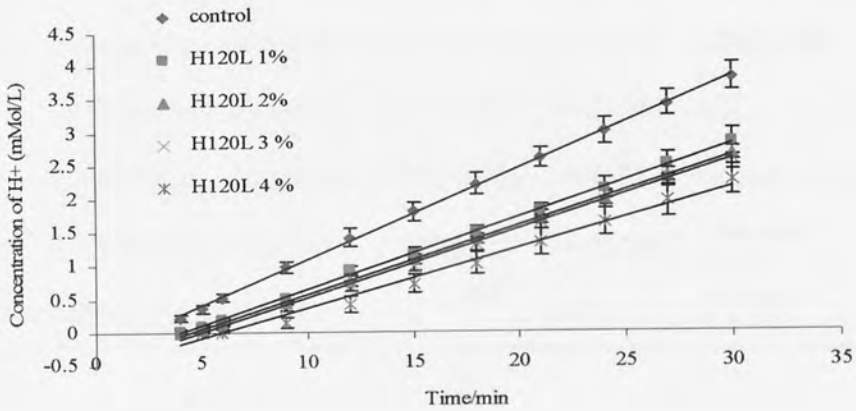
The viscosity of an alginate is related to its concentration with a high concentration leading to an increased viscosity. The effect of concentration of acid diffusion was

investigated in this study. Figure 4.5 shows the results. In Table 4.8 the percentage reduction of acid diffusion was calculated.

a.



b.



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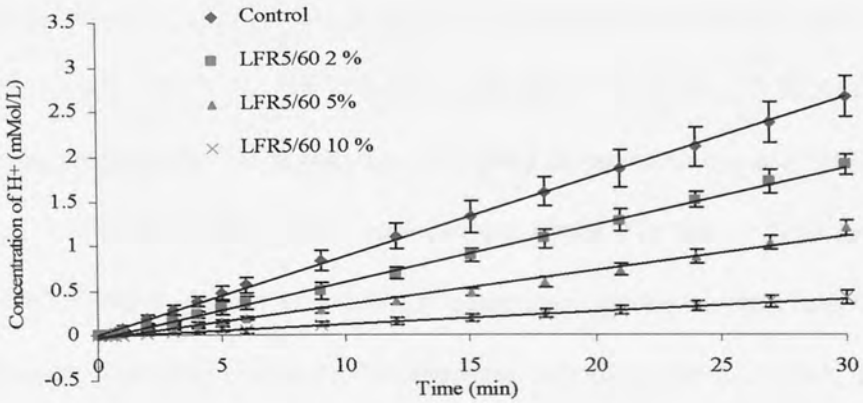


Figure 4.5 The effect of different concentrations of the alginate solution (a =LF120, 1%, 2 %, 3 %, 4 % w/v, b = H120L, 1 %, 2 %, 3 % w/v, c = LFR5/60 2 %, 5 %, 10 % w/v) on acid diffusion (n=4, mean ± s.d.)

Because the viscosity of H120L (4 %, w/v) was too high to allow accurate dispensing of the required volume, no experimental data was obtained. On the contrary, LFR5/60 (1 % w/v) has such a low viscosity that the alginate solution could not form an even layer on top of the membrane, thus there was no data for 1 % w/v of LFR5/60 either.

Table 4.8 The effect of different concentrations of alginates reducing acid diffusion (* acid diffusion rate, † percentage of acid diffusion compared with control, mean ± s.d., n=4)

	LF120		H120L		LFR5/60	
	*mM/min	†%	*mM/min	†%	*mM/min	†%
control	0.137±0.006	100±4.75	0.137±0.006	100±4.75	0.087±0.007	100±8.36
1%	0.114±0.008	83.5±4.75	0108±0.007	79.0±5.34	N/A	N/A
2%	0.104±0.012	76.2±8.85	0.102±0.004	75.1±3.07	0.065±0.007	74.3±7.67
3%	0.099±0.013	73.0±9.36	0.102±0.006	74.8±4.61	0.038±0.003 5 % (w/v)	42.9±3.55
4%	0.090±0.010	65.8±7.17	N/A	N/A	0.014±0.002 10 % (w/v)	15.5±2.47

Table 4.8 highlights the reduction in acid diffusion rate for each alginate compared to the control. Reductions of 17, 24, 27 and 35 % were observed for LF120 at 1, 2, 3 and 4 % w/v. H120L 1 %, 2 %, and 3 % (w/v) showed 21 %, 24 %, 25 % acid diffusion reductions, respectively. No significant difference observed in the acid diffusion rate between LF120 and H120L at the same concentrations. For both LF120 and H120L, acid diffusion rate decreased as solution concentration increased. Although there were no significant differences between neighbouring concentrations (ANOVA, $P > 0.05$), they all significantly reduced acid diffusion compared to the control (ANOVA, $P < 0.05$). For LFR5/60 2 %, 5 %, 10 % (w/v) reductions in acid diffusion rates were 26 %, 57 %, 84 %, respectively. Increasing concentration significantly decreased acid

diffusion (ANOVA, $P < 0.05$). As the concentration of alginate increased there was more solid structure to form the cross-links and reduce the mesh size in the three-dimensional gel. The smaller mesh size retarded the ions' diffusion. The greatest effects were noted with LFR5/60, which has the greatest G content, once again confirming the observation that G units are most important in the formation of alginic acid based gels.

Depths seem to influence acid diffusion to a greater extent than concentration, because increased depth significantly reduced the acid diffusion of LF120 and H120L but increasing concentrations did not show significantly reduced diffusion for both of them. On the contrary for LFR5/60 increased concentrations (2 %, 5 %, 10 %) significantly reduced acid diffusion, but differences in depth did not show a significant change. As stated previously increasing concentration reduces the mesh size in three-dimensional gel, increased depth lengthens the path-length of ions. In the concentration study, H120L and LF120 did not show significant reductions in acid diffusion with increasing concentration whereas LFR5/60 did. The reason is when concentration increases it reaches a level where the mesh size can be significantly reduced and lead to a significant reduction in acid diffusion, beyond this level further increases in concentration do not further reduce the mesh size. H120L and LF120 just increased 1 percent between neighbour concentrations suggesting that the mesh size was already very small, but when LFR5/60 concentrations increased from 2 % to 5 % then to 10 % that significantly influenced the mesh size. For depth increasing, 0.22 mm to 0.44 mm to 0.88 mm all significantly reduced the acid diffusion in both H120L and LF120, but not LFR5/60. That was due to the low viscosity of LFR5/60 at 2 %

(w/v), which was greatly disrupted and a thinner layer formed on the membrane surface when the acid was dispersed.

4.4.1.4 The effect of some commercial formulations (Alginate-based products)

4.4.1.4.1 Comparison of acid diffusion using alginate-based commercial formulations

Acid diffusion through commercial alginate-based products did not give linear concentration versus time plots (Figure 4.6). There are several reasons for this observation. Firstly, the antacid chemicals within the formulation, such as Calcium Carbonate, Sodium Hydroxide, Sodium Hydrogen Carbonate, diffused through the dialysis membrane and increased the pH value in receptor chamber. Secondly, the antacid chemicals reacted with the acid in the donor solution to stop or reduce the acid diffusion through the layer in the first few minutes. Both of the above reasons increased the pH value within the receptor in the first few minutes' diffusion. Thirdly, $CaCO_3$ or $NaHCO_3$ reacted with H^+ to produce CO_2, and the air bubbles in the formulation layer increased the applied layer depth that postponed the time for acid transfer through the layer. These neutralisation reactions continued within the layer, limiting the transfer of hydrogen ions through the layer so no linear relationship existed between acid diffusion and time.

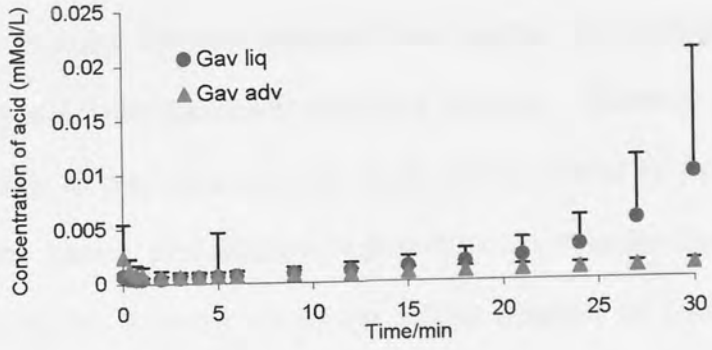


Figure 4.6 Acid diffusion of Gaviscon Advance® and Gaviscon Liquid® over time (n=4, mean ± s.d.)

The lag time of hydrogen ions was defined as the time taken for the diffusion to start throughout the formulations, which could be calculated from intercept divided by gradient of the line (Figure 4.7). This is equal to the time point where the line of fit crosses the x axis.

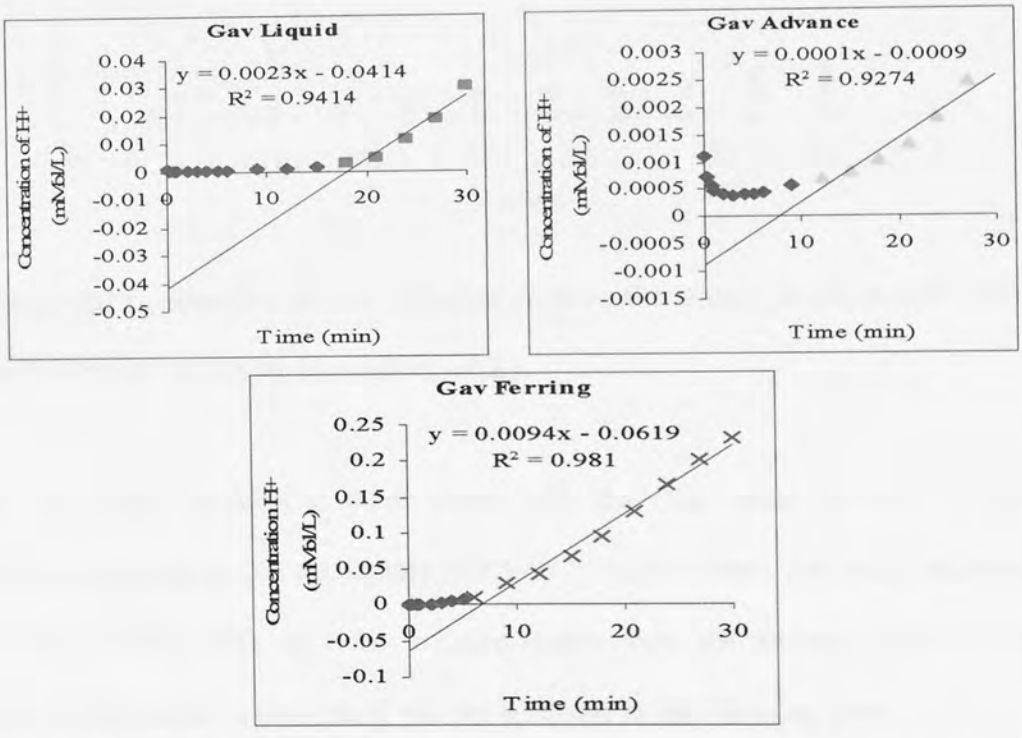


Figure 4.7 Lag time of Gaviscon Advance®, Gaviscon Liquid® and Gaviscon Mikstur® (Gav Ferring) over time.

The lag time was listed as following, Gaviscon Liquid®: 18 minutes; Gaviscon Advance®: 9 minutes; Gaviscon Mikstur®: 6.6 minutes. This indicates that a longer lag time provides better protection from acid diffusion. However, the rate of acid diffusion is also an important factor in the protection offered by the product. From these data the rate of acid diffusion was 0.0023 mM/min for Gaviscon Liquid®; 0.0001 mM/min for Gaviscon Advance®; 0.0094 mM/min for Gaviscon Mikstur®. Gaviscon Advance® presented the lowest diffusion rate, which means during the diffusion process, Gaviscon Advance® provides the best protection against acid diffusion.

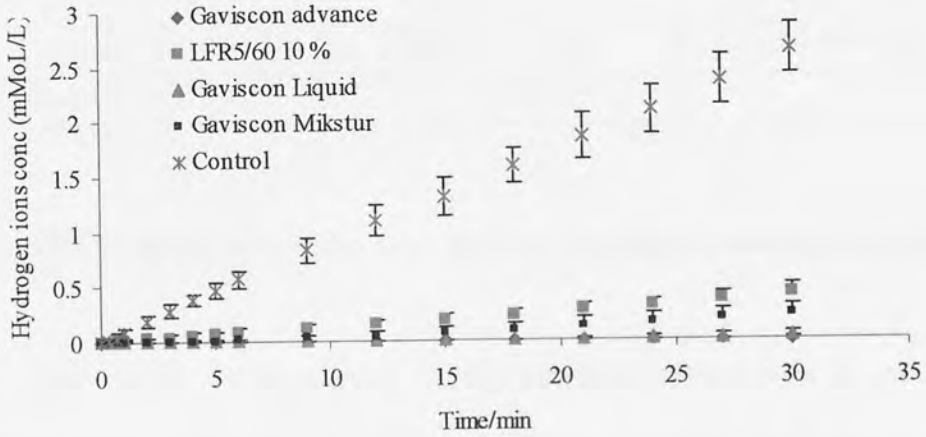


Figure 4.8 Comparison of acid diffusion of three Gaviscon® products with control and LFR5/60 10 % (w/v) (n=4,mean ± s.d.)

As the control gradient is much bigger than the other lines, the lines of three Gaviscon® products and the alginate LFR5/60 10 % (w/v) were very small and almost overlapped (Fig. 4.8). In order to more clearly view the different effects of the commercial products, the control will not be shown in the following graph.

At depth 0.44mm, 10 % LFR5/60 and Gaviscon® products all have a significant effect in reducing acid diffusion. The experimental data are listed in table 4.9. Compared with the control, LFR5/60 10 % (w/v) reduced the acid diffusion by 85 %, Gaviscon Mikstur® reduced diffusion by 90 %, and both Gaviscon Liquid® and Gaviscon Advance® reduced acid diffusion to only 1 - 2 % of the original value.

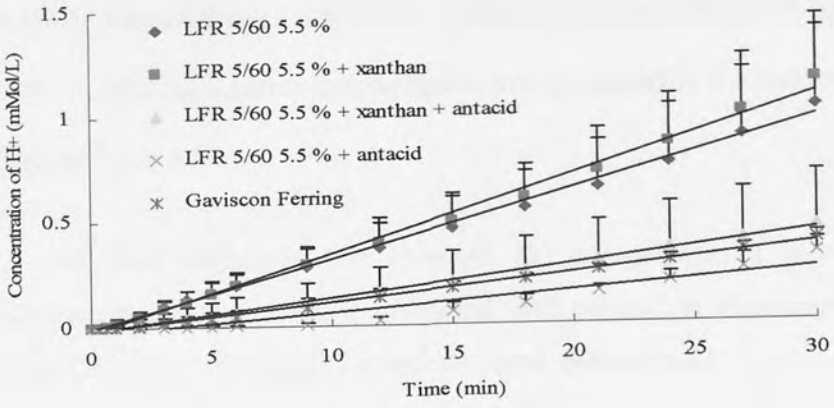
Table 4.9 Acid diffusion rate comparison of Gaviscon® products (mean ± s.d. n=4)

	Diffusion rate (mM/min)	%
Control	0.0873± 0.0081	100 ± 7.90
10 % LFR5/60	0.0139 ± 0.0027	15.92 ± 3.09
Gaviscon Liquid®	0.0023 ± 0.0032	2.63 ± 3.67
Gaviscon Mikstur®	0.0094 ± 0.0030	10.88 ± 3.44
Gaviscon Advance®	0.0001	0.11

4.4.1.4.2 The comparison of the ingredients in Gaviscon® reducing acid diffusion

The capability of the ingredients of alginate-based formulations to reduce acid diffusion were investigated. A solution of alginate; Alginate + thickeners; Alginate + antacids; Alginate + thickeners + antacids; alginate-based commercial products.

a.



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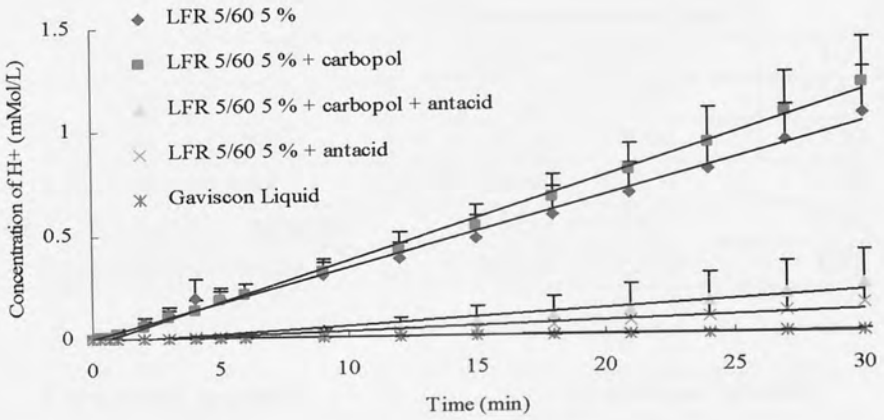

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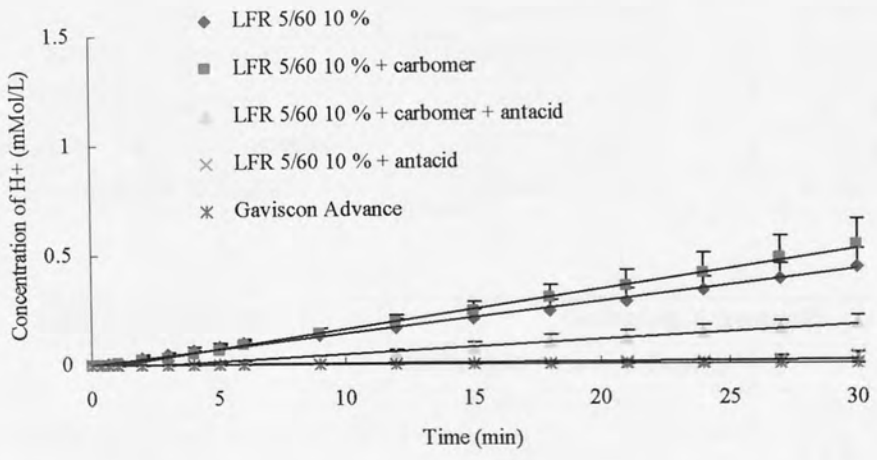


Figure 4.9 Acid diffusion through the formulations of three Gaviscon® products over time a. Gaviscon Mikstur®, b. Gaviscon Liquid®, c. Gaviscon Advance® (n=4,mean ± s.d.)

Alginates reduce hydrogen ion diffusion and antacid chemicals neutralise the acid. Fig. 4.9 shows the acid diffusion profile, the diffusion rate was calculated (as the gradient) and used to calculate a percentage diffusion rate compared to the control; these values are listed in Table 4.10.

Table 4.10 Acid diffusion rate through the ingredients of three Gaviscon® formulations and the percentage compared with control (a. Gaviscon Mikstur®, b. Gaviscon Liquid®, c. Gaviscon Advance® , n=4, mean ± s.d.)

a.

Compared factors	***Gaviscon Mikstur®***	
	Diffusion rate (mM/min)	**%**
Control	0.0934 ± 0.0074	100 ± 7.90
LFR5/60	0.0325 ± 0.0117	34.82 ± 12.53
LFR5/60 + xanthan	0.0368 ± 0.0094	39.42 ± 10.05
LFR5/60 + antacid	0.0095 ± 0.0020	10.18 ± 2.17
LFR5/60 + xanthan+ antacid	0.0151 ± 0.0086	16.12 ± 9.21
Commercial product	0.0129 ± 0.0013	1.38 ± 1.41

b.

Compared factors	***Gaviscon Liquid®***	
	Diffusion rate (mM/min)	**%**
Control	0.0934 ± 0.0074	100 ± 7.90
LFR5/60	0.0336 ± 0.0068	35.99 ± 7.28
LFR5/60 + Carbopol	0.0393 ± 0.0064	42.10 ± 6.86
LFR5/60 + antacid	0.0041 ± 0.0024	4.37 ± 2.58
LFR5/60 + Carbopol+ antacid	0.0073 ± 0.0059	7.84 ± 6.29
Commercial product	1.45E-04 ± 0.0001	0.16 ± 0.14

c.

Compared factors	***Gaviscon Advance®***	
	Diffusion rate (mM/min)	**%**
Control	0.0934 ± 0.0074	100 ± 7.90
LFR5/60	0.0139 ± 0.0027	14.94 ± 0.29
LFR5/60 + Carbopol	0.0178 ± 0.0039	19.07 ± 4.15
LFR5/60 + antacid	0.0006 ± 0.0005	0.61 ± 0.51
LFR5/60 + Carbopol+ antacid	0.0062 ± 0.0014	6.64 ± 1.50
Commercial product	2.00E-05	0.02

Among the three commercial products Gaviscon Advance® showed the best effect to reduce the acid diffusion, just 0.02 % acid diffusion through the formulation compared with the control. The other two products Gaviscon Liquid® and Gaviscon Mikstur® were 0.16 % ± 0.14 %, 1.4 % ± 1.41 % respectively. Of the active ingredients LFR5/60 has the main role in reducing acid diffusion. However, the addition of thickeners (carbomer or xanthan) to all three Gaviscon® products lead to an increase in the acid diffusion rate. When the antacids were added, the acid diffusion rate was reduced again compared to alginate alone. But when all the active ingredients, alginate, carbomer and antacid were mixed together forming a barrier to the acid they were still not as effective as the commercial products. This may be due to overage present in commercial formulations or the addition of flavours and preservatives that may also be beneficial in reducing acid diffusion.

Table 4.11 compares the three formulation strategies for the alginate-based commercial products that were tested.

Table 4.11 Acid diffusion percentage of three Gaviscon® Products (Control = 100 %)

Compared factors	Gaviscon Mikstur® %	Gaviscon liquid® %	Gaviscon advance® %
Control	100 ± 7.90	100 ± 7.90	100 ± 7.90
LFR	34.82 ± 12.53	35.99 ± 7.28	14.94 ± 0.29
LFR + Carbopol/xanthan	39.42 ± 10.05	42.10 ± 6.86	19.07 ± 4.15
LFR + antacid	10.18 ± 2.17	4.37 ± 2.58	0.61 ± 0.51
LFR + Carbopol/xanthan+ antacid	16.12 ± 9.21	7.84 ± 6.29	6.64 ± 1.50
Commercial product	1.38 ± 1.41	0.16 ± 0.14	0.02

4.4.2 Studies on pepsin diffusion

Calibration of the pepsin solution

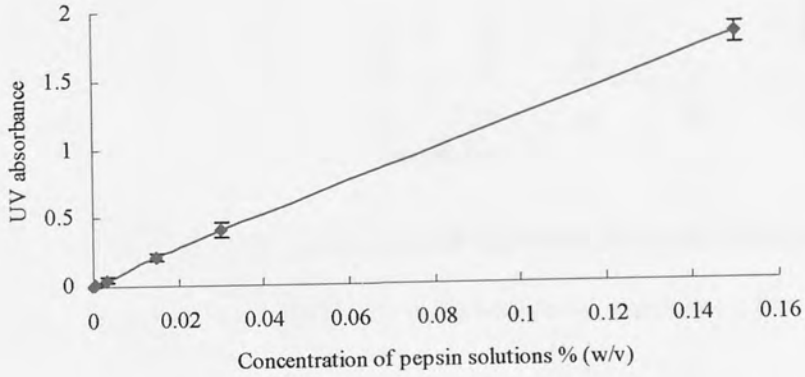


Figure 4.10 Calibration of the acidified pepsin solution, UV absorbance vs. Concentration of pepsin solutions (n=4, mean ± s.d.)

A series of concentrations (0 %, 0.0003 %, 0.003 %, 0.015 %, 0.03 %, 0.15 %, w/v) of acidified pepsin solution were made and the UV absorbance was measured to set up a calibration between concentration and absorbance (Figure 4.10). The concentration of each sample collected at set time points was calculated from the calibration equation : $y = 11.811x + 0.0197$ ($R^2 = 0.9992$) (calculated from Figure 4.10)

The rate of pepsin diffusion through the alginate layers was examined. 0.1 mL of each alginate at 2 % w/v was used and the rate of pepsin diffusion was measured, the results are shown in Fig. 4.11.

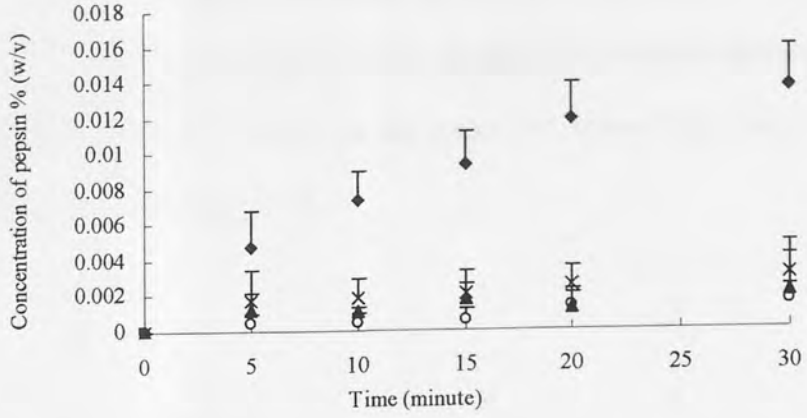


Figure 4.11 Comparison of the rate of pepsin diffusion for three alginates (2 %, w/v) (♦ = control; ○ = LF120; ▲ = H120L; x = LFR5/60 (n=4, mean ± s.d.)

The rate of pepsin diffusion was not a linear phenomenon, so the area under the curve (AUC) was used to compare the extent of pepsin diffusion for the test solutions. However, the results above show that all alginates demonstrated some resistance to pepsin diffusion compared to the control. Table 4.12 shows the results for the area under the curve (AUC) for all the alginates examined, the control value was normalised to 100 % and the relative areas are shown as a percentage of this control value. GraphPad Prism® 3.0 was used to calculate the area under the curve for each pepsin diffusion line.

Table 4.12 Area under the curve (AUC) of the pepsin diffusion through the three alginates within 30 minutes and transferred the AUC to percentage, control = 100 %, (n=4, mean ± sd.)

	AUC	%
control	0.262 ± 0.045	100 ± 17.28
LF120 2 %	0.026 ± 0.013	9.88 ± 5.00
H120L 2 %	0.039 ± 0.013	14.92 ± 4.85
LFR5/60 2 %	0.062 ± 0.022	23.54 ± 8.51

The results show that all alginates demonstrated significantly reduced pepsin diffusion (ANOVA, $P < 0.05$), however, there were no significant differences between the three alginates tested (ANOVA, $P > 0.05$). A bar graph was drawn from table 4.12 shows these results more clearly (Fig. 4.12).

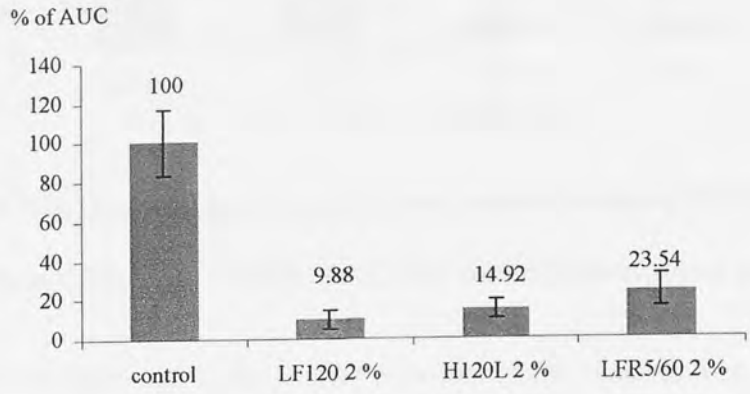


Figure 4.12 Percentage comparison of the area under the curve (AUC) for pepsin diffusion for the three alginates examined (n=4, mean ± s.d.)

2 % w/v LF120 was chosen as an example to study the effect of the aqueous alginate with different concentrations and different layer depths in reducing pepsin diffusion.

In a study of concentration, 3 concentrations of LF120 solution (1 %, 2 %, 4 %, w/v) were used. The analysis method was as same as before. The data and graph are presented in Table 4.13 and Fig. 4.13.

Table 4.13 Area under the curve (AUC) of the pepsin diffusion through alginate (LF120) layer at different concentrations within 30 minutes and transferred the AUC to percentage, control = 100 %, (n=4, mean ± sd.)

	AUC	%
control	0.262 ± 0.045	100 ±17.28
1%	0.086 ± 0.024	32.81 ± 9.19
2%	0.036 ± 0.012	13.74 ± 4.43
4%	0.035 ± 0.022	13.16 ± 8.24

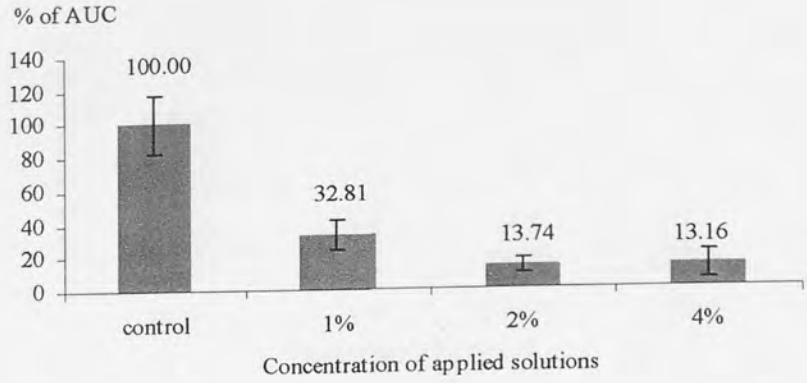


Figure 4.13 Percentage comparison of the area under the curve (AUC) for pepsin diffusion through alginate (LF120) at different concentrations, (n=4, mean ± s.d.)

As with the acid diffusion study, further increases in the concentration of LF120 did not further reduce the diffusion of pepsin between 2 and 4 % w/v; this may be due to the mesh size within the alginate already being of a small size and no further decreases occurred at these elevated concentrations.

In the study of depths, 3 concentrations of LF120 2 % (w/v) solutions (0.22 mm, 0.44 mm, 0.88 mm) were used in this study, the data and graph were presented in Table 4.14 and Figure 4.14.

Table 4.14 Area under the curve (AUC) of the pepsin diffusion through alginate (LF120) layer at different depths within 30 minutes and transferred the AUC to percentage, control = 100 %, (n=4, mean ± s.d.)

	AUC	%
control	0.262±0.045	100±17.28
0.22mm	0.088±0.020	33.69±7.55
0.44mm	0.032±0.010	12.02±3.85
0.88mm	0.036±0.011	13.77±3.97

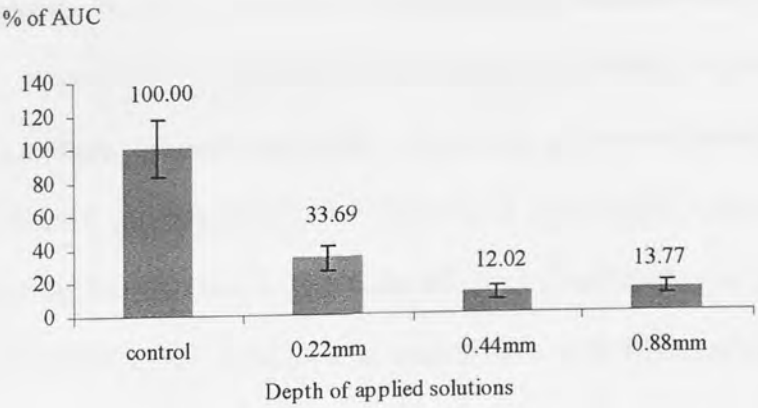


Figure 4.14 Percentage comparison of the area under the curve (AUC) for pepsin diffusion through alginate (LF120) at different depths, (n=4, mean ± s.d.)

An increase in alginate concentration from 1 % to 2 % (w/v) lead to a significant reduction in pepsin diffusion from 32.81 % ± 9.19 % to 13.74 % ± 4.43 % ($p<0.05$). However a further increase in alginate concentration did not reduce pepsin diffusion 13.16 % ± 8.24 % at 4 % (w/v). Increasing the depth of the alginate layer applied had a similar effect; doubling the depth of the layer from 0.22 mm to 0.44 mm lead to a significant reduction in pepsin diffusion from 33.69 % ± 7.55 % to 12.02 % ± 3.85 %, however a further increase to 0.88 mm did not significantly affect pepsin diffusion 13.77 % ± 3.97 ($P>0.05$).

4.5 CONCLUSIONS

This chapter is linked to chapter 2 Retention study, which demonstrated that aqueous alginate was retained on oesophageal mucosa for up to 30 minutes forming a protective bandage. If the bandage can reduce the two harmful factors, acid and pepsin, contacting the oesophageal surface, damage to the oesophageal mucosa will be reduced.

Of the 3 alginates, H120L, LF120, LFR5/60 at the same concentration 2 % (w/v) and same layer depth 0.44 mm, LFR5/60 showed the best ability to reduce the acid diffusion, but there was no statistically significant difference between the three alginates (ANOVA $p>0.05$). H120L and LF120 had very similar effects in reducing acid diffusion. In the study that looked at the effect of concentration of acid diffusion, the ability of both of H120L and LF120 to reduce the acid diffusion slightly increased as the concentration of solutions increased but there was no significant difference between neighbour concentrations. The viscosity limitation occurred when the concentration of H120L was more than 3 % (w/v) and LF120 was more than 4 % (w/v), as the solution was unable to spread and form a homogenous layer, thus no higher concentrations were evaluated. The concentrations of LFR5/60 investigated were 2 %, 5 %, 10 % (w/v) and the ability to reduce acid diffusion significantly improved as concentration increased.

When the depth of the layer was examined, both H120L and LF120 significantly reduced the acid diffusion as depths increased ($P<0.05$) at the same concentration 2 % (w/v), LFR5/60 reduced acid diffusion as layer depths increased, too, although there was no significant difference between neighbour depths.

The commercial products, Gaviscon Mikstur®, Gaviscon Liquid®, Gaviscon Advance®, had much better ability to reduce acid diffusion compared to LFR5/60 at 10 % (w/v), which was the best in acid diffusion reduction of the three alginates at all concentrations at a depth of 0.44 mm. Gaviscon Advance® had the best effect in reducing acid diffusion compared to the other two alginate-based commercial products.

The alginate, thickener and antacids were evaluated from the alginate-based commercial products as barriers to acid diffusion. Alginate formed the main function in acid diffusion reduction, about 65 % reduction in Gaviscon Mikstur®, 64 % in Gaviscon Liquid®, 85 % in Gaviscon Advance®. The second main factor was antacids, an additional 18 % reduction in Gaviscon Mikstur®, 19 % in Gaviscon Liquid®, 9 % in Gaviscon Advance®. Thickeners are present to increase the viscosity of the formulation and aid in the suspension of antacids. However, when the thickeners were added, the ability of the formulations to reduce acid diffusion was reduced. The possible reason is that the ions in the thickener reduce the viscosity of the alginate solution, which increased acid diffusion.

The pepsin diffusion did not have a linear relationship with time demonstrating this is not an even diffusion. Because of the high molecular weight of pepsin, 35 kDa, the pepsin molecules were quite easily trapped within the alginate matrix. All three alginates significantly reduced pepsin diffusion. When the alginate concentration changed from 1 % to 2 % (w/v) pepsin diffusion was significantly reduced. Further alginate concentration increase to 4 % (w/v) did not further reduce pepsin diffusion. The same situation happened with increasing depth. When depths changed from 0.22mm to 0.44mm, pepsin diffusion was significantly reduced; thicker depth 0.88mm did not significantly change the pepsin diffusion.

In summary, aqueous alginates are very effective in reducing acid and pepsin contact the oesophageal mucosa thus they have potential to protect the oesophagus from the damage of the gastric refluxate.

CHAPTER 5 CELL CULTURE

5.1 INTRODUCTION

GORD (Gastro-oesophageal reflux disease) is believed to lead to morphological changes in cells that line the oesophagus due to damage by acid and pepsin present in gastric refluxed materials. A morphological alteration in oesophageal epithelial cells to those found in gastric epithelium is termed Barrett's oesophagus. Previous work (Batchelor H.K. et al. 2002) has shown that alginate is able to adhere to porcine oesophageal mucosal for a period of up to 60 minutes and the study in chapter 2 showed similar retention capability. The diffusion rate of both acid and pepsin was significantly decreased by the presence of an alginate layer, as demonstrated in chapter 4. This study examined the ability of alginates to coat individual cells and protect them from the reflux components including pepsin and acid.

Cell culture

Immortalized cell lines have been used as drug absorption models for many years and aid in the understanding of drug permeability mechanisms (Artursson, 1990). Most cell lines were derived from carcinoma cells because of their rapid growing speeds and immortalization. In this study Caco-2 cells were used for their convenience, easy accessibility and robust nature.

Caco-2 cells are derived from a human colonic adenocarcinoma; they differentiate into a cell monolayer barrier representative of a small intestinal columnar epithelium. Caco-2 cells are generally used for permeability screening of a new drug, as an indicator of the *in vivo* intestinal absorption of compounds (White, 2000). The drug permeability across the Caco-2 cell layer is determined by growing the cells on a membrane placed between an insert plate and a feeder tray. When the cell monolayer is ready, the test is initiated by adding compounds to the apical side of the cell layer. After a chosen period of time, the basolateral side is sampled and analysed.

In this study the Caco-2 cells were not used as a permeable monolayer for the study of new drug diffusion and absorbance, but as a cell suspension, which was mixed with liquid alginate to test if alginate can coat on individual cells and protect them from acid and pepsin, the main compounds in gastric reflux. Caco-2 cells do not secret mucus. So if there is a layer coating the cells it is not secreted by the cell itself (Artursson et al, 2001).

5.2 MATERIALS AND APPARATUS

5.2.1 Materials

Three liquids composed of the cell working media were all purchased from Invitrogen, UK. They were Foetal calf/bovine serum (FCS); Minimum essential medium (MEM) containing non essential amino acid liquid with L-glutamine; Dulbecco's modified eagle medium (DMED) containing high glucose with L-glutamine. 100 mL FCS and 6 mL MEM were added to 500 mL of DMED under aseptic conditions to give a working supply of complete media. The media was used for the ongoing culture which is

maintained in the flasks. This media was marked with X and stored at temperature 0 – 4 °C.

Trypsin EDTA (Invitrogen, UK) is a mixed liquid of 0.25 % Trypsin with 0.2 % EDTA. Trypsin EDTA liquid was used to remove the confluent growing cells from the flask wall for harvesting the cells that was used in experimental operations or reseeding cells in new flasks.

Dulbecco's phosphate buffered saline (PBS) was used for media washing in the procedure of cell reseeding and cell pellet re-suspending in the experiments. 2 % Trigene and 70 % EtOH was used to clean the inside of the class II cabinet.

0.4 % w/v Trypan blue (Sigma, UK) was dissolved in physiological saline. This solution was used to stain dead cells in the cell viability assay.

5.2.2 Apparatus

5.2.2.1 Class II cabinet and incubator

Most of the cell work was performed in a sterile cabinet (GELAIRE® BSB4A). Before the cell work the UV light door was taken off and the fan was activated to provide sterile air for at least 10 minutes. The cabinet was then cleaned using 2 % Trigene followed by 70 % EtOH and all surfaces inside the cabinet were wiped with wet sterile tissue. The base was cleaned as often as need with 70 % EtOH while the cabinet was used. After the cell work the same cleaning procedure was performed. After wiping the surfaces with wet sterile tissue; 70 % EtOH spray was used over all of the cabinet

surfaces, this was sprayed from a distance of 30cm to complete the sterilisation process, then the cabinet was left for around 15 minutes to dry under sterile conditions. The UV light instrument was installed to cover the cabinet whilst the cabinet fan was off. The UV light was immediately switched on for another 15 minutes sterilisation after the fan was turned off.

The cell incubator used in this study was model LEEC MKII, the flasks of cultured cells were kept in a humidified environment at 5 % CO_2 and 37 °C within the incubator.

5.2.2.2 Counting chamber

Structure of Counting Chamber

A device used for cell counting is called a counting chamber. The most widely used type of chamber is called a haemocytometer as it was originally designated for performing a blood cell counts. A counting chamber can only determine cell density of a suspension spectrophotometrically, but it does not allow an assessment of cell viability and distinguish cell types. Figure 5.1 schematically represents the structure of a counting chamber.

To prepare the counting chamber the coverslip and the mirror-like polished surface of the coverslip support are carefully cleaned with lens paper. Coverslips for counting chambers are specially made and are thicker than those for conventional microscopy, since they must be heavy enough to overcome the surface tension of a drop of liquid. The coverslip is placed over the counting surface prior to putting on the cell

suspension, which is introduced into one of the V-shaped wells with a Pasteur pipette. The area under the coverslip fills by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered. The chamber then is placed on the microscope stage and the counting grid is brought into focus at low power.

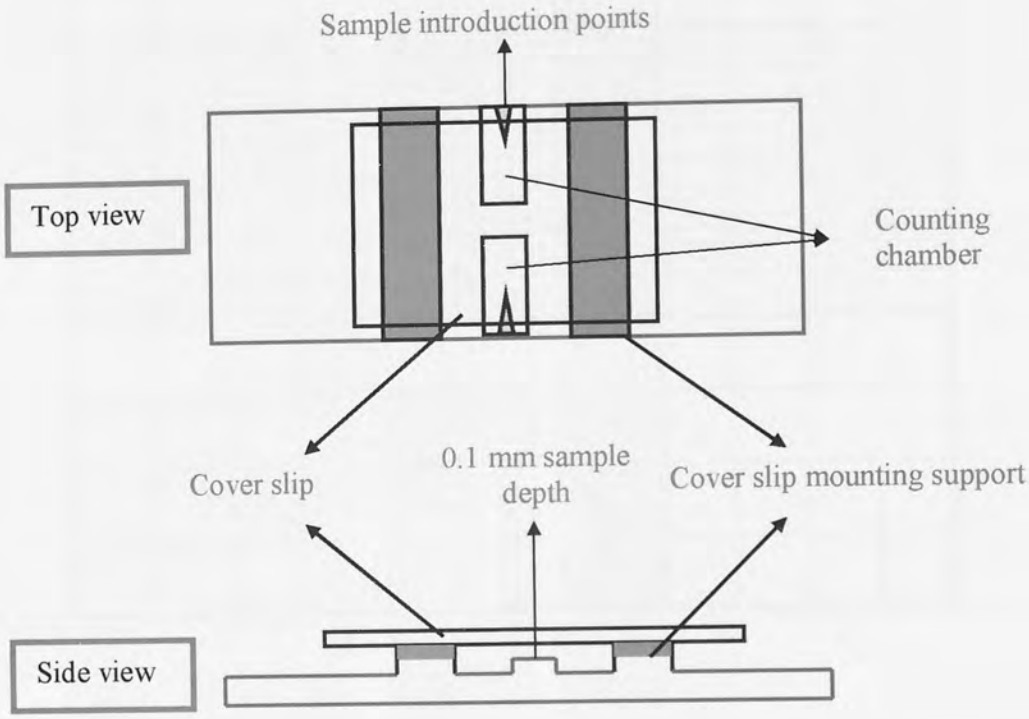


Figure 5.1 Schematic representation of Counting Chamber

Counting Grid

The counting grid is located on the mirror-like surface. An entire grid on a standard haemocytometer can be seen at 40x magnification. The main divisions separate the grid into a total area of 9 mm^2 (3 x 3 mm) shown in Figure 5.2, the depth of the chamber is 0.1 mm. Thus the entire counting grid lies under a volume of 0.9 mm^3.

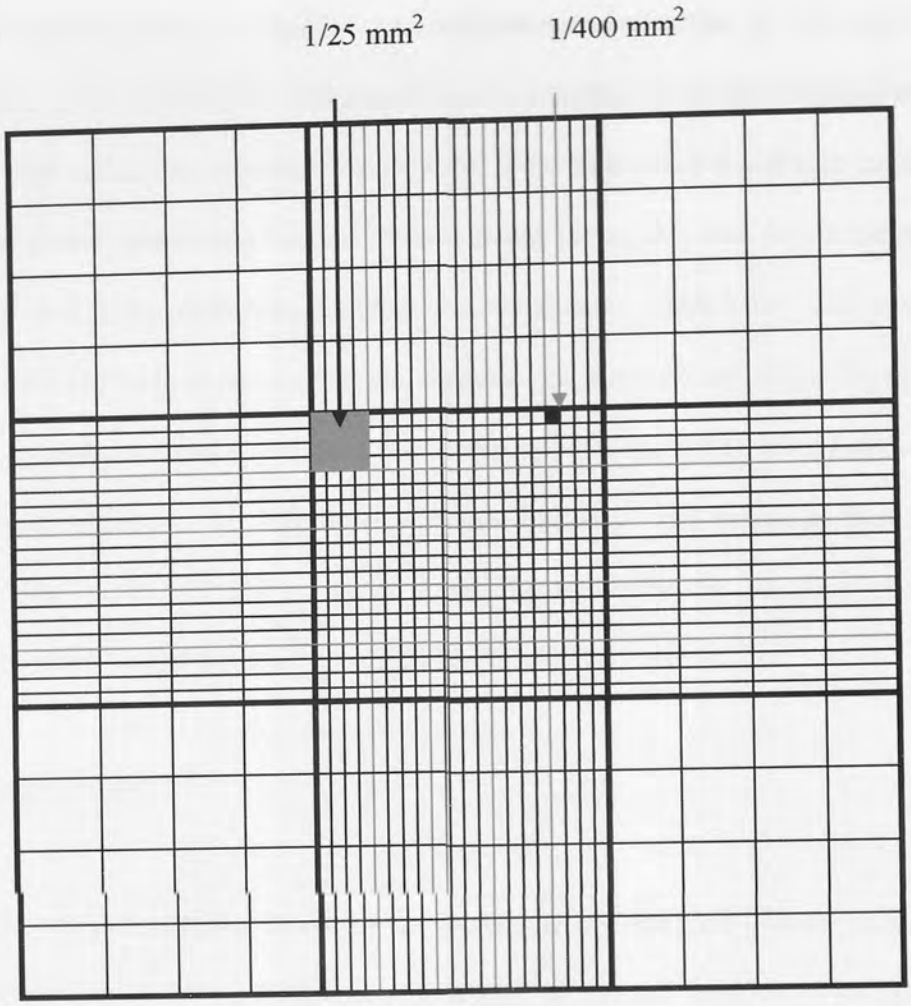


Figure 5.2 Schematic representation of counting grid

Method of Cell Counting and calculation

Cell suspensions should be diluted enough so that the cells do not overlap each other on the grid. To perform the count, determine the magnification needed to recognize the desired cell type. For large cells this may mean counting the four large corner squares and the middle one. For cells that overlap a ruling, count a cell as "in" if it overlaps the top and right ruling, and "out" if it overlaps the bottom and left ruling.

The method, using a standard haemocytometer determines a final cell count in cells/mL, firstly divide the total count by 0.1 (chamber depth) then divide the result by the total surface area counted. For example if the total count is 125 cells in all the four large corner squares and the middle one, divide 125 by 0.1, then divide the result by 5 mm^2 that is the total area counted, the equation is 125/0.1/5 = 250 cells/mm^3 = 250,000 cells/mL. If the original cell suspensions were diluted to get the cell density low enough for counting, the final count should be multiplied by the dilution factor. In the example above, the cell suspension was diluted 10 fold before perform counting. The final count 250,000 cells/mL should be multiplied by 10. Then the original suspension concentration is 10 x 250,000 = 2,500,000 cells/mL.

5.3 METHODS

Cells are quite fragile, therefore the procedures within cell culture must be very delicate. The environment and apparatus used in the cell work must be cleaned and solutions are aseptic. In this study the procedures included: thawing cells; preparations prior to cell work; procedure for trypsinising, counting, and seeding cells; changing media; cell viability assay. These detailed procedures are described below.

5.3.1 Procedure of thawing cells

To initiate the cell work, cells were collected from a liquid nitrogen store and placed on ice whilst transferring to the tissue culture lab. Immediately, the cells were warmed in a 37 °C water bath, shaking occasionally. The cells were thawed until the last crystal melted. Under aseptic conditions, the cells were transferred to a centrifuge tube. The suspension was mixed with approximately 10 mL cold complete medium. Then the

cell suspension was centrifuged at 300 "RCF" (About 1177 rpm) for 5 minutes. The supernatant was decanted and the cells were gently re-suspended in 10 mL complete working media. The cells were counted (as described in 5.2.2) and seeded into 25 cm^2 flasks at a density of 2 x 10^5 cells/flask in 7 mL media. The media was then changed every two days (The procedure is described in 5.3.3). When the cells looked to be confluent trypsinisation of the cells was carried out and the cells were then re-seeded into 75 cm^2 flasks in 25 mL media at a density of 3.5 x 10^5 cells/flask (the procedure is described in 5.3.4).

5.3.2 Preparations before working with cells

The materials were collected prior to use; the complete medium and PBS solution were put into a water bath (37 °C) and kept there at least for 30 minutes or if trypsinisation was performed Trypsin EDTA solution needed to be placed in a water bath beforehand. The cells were inspected under the inverted microscope (Nikon TMS) to check that they were ok and ready for the next procedure.

The exterior of any materials, such as bottles or packets of flasks or culture plates and equipment like the strippetor were cleaned with 70 % EtOH. Following cleaning, they were placed in the hood immediately. Care was taken to clean thoroughly anything that was wet, including the bottles just removed from the water bath. It was best to place things to either side and towards the back of the hood. This leaves the central area uncluttered which assists the laminar flow of sterile air.

When transferring relevant ongoing culture flasks directly from the incubator to the class II cabinet, each flask was sprayed with 70 % EtOH before placing in the cabinet.

5.3.3 Procedure for changing media

Every two days the media in the flasks was changed. A Vacuset waste bottle located beside the cabinet was used for collecting the waste media. Carefully a sterile Pasteur pipette was removed and attached to the Vacuset handset.

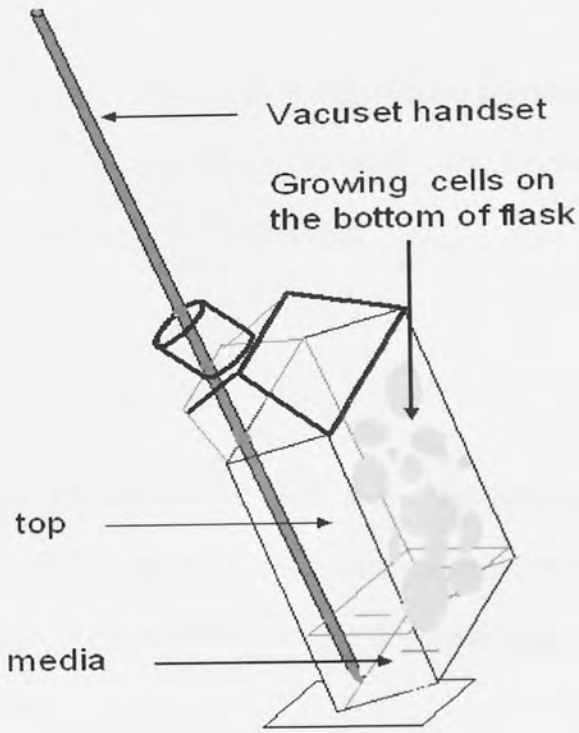


Figure 5.3 Schematically represented the procedure of media change

The flask was placed in the centre of the hood towards the back. The flask was tilted so that the media was on the "top" of the flask away from the cells (schematically represented in Figure 5.3). Carefully, the old media was removed.

The media bottle was placed in the middle of the hood and 25 mL of media was aliquoted into the flask, making sure the pipette did not touch the flask. The liquid was carefully dropped from the flask neck and gathered on the "top" of the flask away

from the cells (same position as in Figure 5.3). The flask was then sealed and the media was allowed to cover the cells. Once all the media had been changed, all the flasks were sprayed with 70 % EtOH and taken to the incubator. Making sure to label every flask on the top side to easily read them without disturbing the cells.

5.3.4 Procedure for trypsinising, counting and reseeding the cells in new flasks

While cells were growing in flasks they became confluent, they need to be trypsinised and reseeded into new flasks. Generally the trypsinisation and reseeding was performed once a week for Caco-2 cells.

Trypsinisation

Visual inspection was used to determine if the cells were ready for trypsinisation, using a microscope. 60 – 80 % confluence meant it was time for trypsinisation. Media, PBS solutions, Trypsin EDTA were put in a water bath at 37 °C for 30 minutes before this procedure.

The first few steps were the same as the media change: check the Vacuset waste bottle was relatively empty with sterile agent inside the bottle, turn on the vacuum pump; put on gloves and sterilise them; attach a sterile Pasteur pipette to the Vacuset; loosen all lids of bottles and flasks inside of the hood; remove the old media using Vacuset as described in 5.3.3.

A sterile pipette was attached to the Strippetor and a 10 mL aliquot of PBS was carefully dispensed into the flask. The liquid was gently dropped into the neck of the

flask so that it gathered against the "top" away from the cells; the lid was put on the flask and gently shaken to move the PBS around the whole of the flask, making sure every surface was rinsed. The flask was tilted so that the dirty PBS flowed away from the cells and the liquid was removed using the Vacuset. This wash was performed at least three times. It is very important to remove all of serum, because any remaining serum inactivated the Trypsin.

When the cells were free of the old media and PBS, a new sterile pipette was attached to the Stripettor; 5 mL of Trypsin EDTA solution was aliquoted into the flask; the flasks were capped and put back in the incubator and the flasks were checked after 2 - 3 minutes. When it was observed that cells were coming off the flask and into the suspension, the flasks were transfered to the hood; the flask was tapped against the palm to wash the Trypsin over the remaining layers of cells. Using a new sterile pipette attached to Stripettor 10 mL medium was aliquoted into a trypsinised flask, using the Stripettor to repeatedly flush the flask to disperse any remaining cells. This cell suspension was then transferred for experimental use or for reseeding as described in the following paragraph.

Reseeding

The total seeding density for a 75 cm^2 flask was 3.5 x 10^5. Using a clean pipette and Strippetor, the correct volume of cell suspension was aliquoted, in this study 2 mL cell suspension was dispensed in a new flask after trypsinisation, then fresh media was added into the flask to give a total volume of 25 mL. The flasks were then gently mixed and left so that the cells gathered across the "bottom" of the flasks.

When finishing reseeding all new flasks were labelled with (a) cell types, (b) passage number, (c) date and (d) operator name. Then the flasks were sterilised with 70 % EtOH and placed in an incubator.

5.3.5 Viability assay of cells

Viability assays measure the percentage of a cell suspension that is viable (Freshney, 1994). This is generally accomplished by a dye exclusion stain, where cells with an intact membrane are able to exclude the dye while the dead cells without an intact membrane take up the colour agent. The dye used for exclusion stain is usually trypan blue but erythrosine and naphthalene black have also been used (West et al, 2000; Cairns & Hay, 1994). In this study trypan blue was used as staining agent. The cell staining procedure is described in the following paragraph.

A cell suspension was prepared for the assay (about 1 x 10^6 cells/mL). The suspension was diluted 1:1 using a 0.4 % trypan blue solution. The diluted suspension was introduced into the counting chamber and left for 1 minute (Do not leave longer as viable cells may die and begin to take up the dye). The number of stained cells was counted as well as the total number of cells (the counting method was described in 5.2.2). The calculated percentage of unstained cells represents the percentage of viable cells.

5.3.6 Designated experimental procedure

On the day of reseeding cells, the cell suspension was collected into centrifuge tubes. Each tube contained 10 mL suspension. The centrifuge used in the study was model

MSE Mistral 3000i. The cell suspension was centrifuged at 300 RCF (1177 rpm) or 500 RCF (1520 rpm) for 5 minutes or longer period (as stated), and the supernatant was discarded, a set concentration of alginate and /or damage factor, eg. hydrochloric acid in a volume of 5 mL was added. This cell suspension was stirred and stored in a water bath for a set period. The suspension was then centrifuged again at 300/500 RCF for 5 minutes or longer. The pellet was washed using PBS solution at least 3 times to remove the excess polymer (alginate) and/or the damage factors (acid and pepsin). The PBS washing procedure involved the addition of 10 mL PBS into the centrifuge tube that held the cell pellet and re-suspending the cells then a second centrifugation at 300/500 RCF for 5 minute, discarding the supernatant. After the final wash the cells were re-suspended in fresh PBS and incubated for 30 minutes prior to assessing the cell viability.

5.4 RESULTS AND DISCUSSIONS

5.4.1 Effect of alginate concentration

Alginate H120L 2 % w/v, LF120 2 % w/v were prepared to mix with a cell pellet to study whether alginate can protect the cells from acid. When alginate liquids were added into a cell pellet, even under very vigorous stirring the alginates did not mix with cells because of the high viscosity of the alginate liquid. So the concentration of alginate 2 % w/v of H120L and LF120 was not used in this study. This result suggested that the viscosity of liquid needed to mix with cell pellet had to be very low.

5.4.2 Acid damage study – effect of pH2

pH 2 hydrochloric acid was used to investigate if Caco-2 cells can endure this pH. The challenged cells were kept in acid, a control was kept in same volume of PBS. Both

tubes were kept in a water bath at 37 °C for 1 minute. The centrifuge speed was set 300 RCF for 5 minutes.

Results showed that no cells survived in pH 2 acid. The density of cells in control was 0.38 x 10^5 cells/mL, which suggested that Caco-2 can not survive at pH 2.

5.4.3 Acid and pepsin damage study – effect of LFR5/60 at pH3

LFR5/60 1 % w/v was chosen due to its low viscosity of 7.49×10^{-3} Pa.s at shear rate 10 s^{-1}; pH 3 hydrochloric acid and acidic pepsin 0.1 % w/v in pH 3 acid were used as challenge factors to cells. The cell suspensions mixed with all above solutions were kept in water baths 37 °C for 3 minutes and centrifuged at 500 RCF for 5 minutes. The results are listed in Table 5.1

Table 5.1 Caco-2 cell viability under different challenge factors (LFR5/60 as protection liquid)

Experimental factors	**Cell Viability %**
Control	59 %
LFR5/60 1 % + Acid pH 3	0 %
LFR5/60 1 % + Acid pH 3 + pepsin 0.1 %	3 %
Acid pH 3	0 %
Acid pH 3 + pepsin 0.1 %	0 %

This data show that the control viability was approximately 60 % of the starting concentration thus the centrifugation may cause some cell loss. The alginate was not effective in protecting the cells from the acid damage although some protection was

observed in the presence of both acid and pepsin; however, this result was not repeated due to the extensive preparation work and the disappointing preliminary results.

5.4.4 Acid and pepsin damage study - effect of H120l at pH 3

H120L 0.2 % w/v was prepared; all the other experimental factors were the same as above (Section 5.4.3) except the duration of centrifuge was set at 30 minutes. Because the viscosity of 0.2 % H120L was still very high, 5 minutes centrifugation was not enough to push the cell pellet down to the bottom of centrifuge tube. In order to avoid many cells being discarded with the supernatant, the centrifuge duration was extended to 30 minutes. The results are listed in Table 5.2

Table 5.2 Caco-2 cell viability under different challenge factors (H120L as protection liquid)

Experimental factors	**Cell Viability %**
Control	15 %
H120L 0.2 % + Acid pH 3	0 %
H120L 0.2 % + Acid pH 3 + pepsin 0.1 %	0 %
Acid pH 3	0 %
Acid pH 3 + pepsin 0.1 %	0 %

Both above data of the effects of LFR5/60 at 1 % w/v and H120L at 0.2 % w/v at pH 3 shown in Table 5.1 and 5.2 suggest that Caco-2 cells can not survive at pH 3. There were two items that were different for both studies. First, the duration of centrifuge: Data (LFR5/60) vs. Data (H120L) = 5 minutes vs. 30 minutes, which may lead to the difference in cell viability in the two controls. Centrifugation for 5 minutes showed a higher cell viability (59 %) than that for 30 minutes, 15 %. This indicates that the centrifuge itself has the potential to kill Caco-2 cells at 500 RCF. Secondly, cells

mixed with LFR5/60 under the attack of acidic pepsin, had a cell viability of 3 %, which may suggest that LFR5/60 has the potential to protect the individual cells from acid and pepsin attack.

As the data showed that the Caco-2 cells could not survive at pH3; pH 4 and 5 were used in the next group study. As pepsin loses activity when beyond pH 3.3 (Tobey et al, 2001), no acidic pepsin solution was used in the following study.

5.4.5 Examination of the effect of acids at pH 4 and 5 (group 1)

Due to a shortage of cells on this experimental day, only two tubes of cell suspension were used in this study. Both cell suspensions was incubated in acid pH 4 and pH 5 and kept at 37 °C in a water bath for 3 minutes, then centrifuged at 300 RCF for 5 minutes. The cell viability is listed in Table 5.3.

5.4.6 Examination of the effect of acids at pH 4 and 5 (group 2)

A control tube was also measured that contained cells within PBS on another experimental day while the other conditions were same as group 1. A comparison is shown in Table 5.3.

Table 5.3 Cell viability in hydrochloric acid (pH 4 and pH 5)

	Control	Cell viability pH 4	pH 5
Group 1	NA	23 %	35 %
Group 2	100 %	42 %	41 %

The results above demonstrate that Caco-2 cells have some ability to survive at pH 4 and pH 5 acid, although their natural habitat is in the intestinal environment at approximately pH 7.

5.4.7 Examination of the effect of acids at pH 4 and 5 (group 3)

In investigating the potential of LFR5/60 in protecting cells from acid injury, LFR5/60 1 % w/v was introduced in this study, under the challenge of pH 4. Due to the increase in liquid viscosity, the centrifuge duration was increased to 15 minutes at 500 RCF. The other factors were the same as those in section 5.4.5, incubated in 37 °C water bath for 3 minutes. The results are listed in Table 5.4.

Table 5.4 Cell viability in LFR5/60 1 % liquid challenged by hydrochloric acid pH4

	Control	Cell viability pH 4	LFR5/60 1 % with acid pH 4
Group 3	17 %	0 %	25 %

Table 5.4 gives a quite different result from Table 5.3. The cell viability in pH 4 was zero. The control is also very low, just 17 %. The difference in the procedure between these studies is the centrifuge speed and duration. This study used a centrifugation process that lasted 15 minutes at 500 RCF, whilst the previous study (5.4.6) used 5 minutes at 300 RCF, which suggested again that the centrifuge speed and duration may be key reasons for the differences in viability of Caco-2 cells. 25 % cell viability in LFR5/60 liquid challenged by pH 4 acid indicated that when alginate LFR5/60 1 % w/v was mixed with Caco-2 cells it has the potential to protect cells from acid.

5.5 CONCLUSIONS

The effect of acid at pH 2 and 3 indicated that Caco-2 cells can not survive at acid pH 2 and 3, but yet at pH4 and 5 Caco-2 cells have limited viability. Caco-2 cells are derived from a human colonic adenocarcinoma whose pH living environment is more than 7, yet pepsin, a main role in gastric contents can not be included in the study as gastric pepsin loses activity when the pH value is more than 3.3 (Tobey et al, 2001).

H120L and LF120 at a concentration of 2 % w/v were generally used in the retention study (Chapter 2) and diffusion study (Chapter 4). However, this study found that this concentration could not be used in this study because of their high viscosity. But even with very low concentration of alginate the viscosity was still too high compared with a water solution. In order to get the cell pellet down to the bottom of the centrifuge tube, the centrifuge duration had to be set much longer than the general period time of 5 minutes, such as in the effect of H120L 0.2 % w/v at pH 3 (Section 5.4.4) the centrifugation lasted 30 minutes. This duration reduced the cell viability from 59 % (centrifuge for 5 minute in 5.4.3) to 15 % (centrifuge for 30 minutes in 5.4.4). The centrifuge speed had to be increased from 300 to 500 RCF, like in the effect of pH 4 and 5 (5.4.6 and 5.4.7), when LFR 1 % was added as protective factor the centrifuge duration and speed had to extend from 5 minutes at 300 RCF (5.4.6) to 15 minutes 500 RCF (5.4.7), which reduced the cell viability from 100 % (5.4.6) to 17 % (5.4.7).

So a contrary situation appeared, that if alginate, even at very low concentrations, was added, the centrifuge duration or speed had to be improved to get the required dense cell pellet, these procedures affected the cell viability thus the damage factors could not be assessed. At low concentrations, alginates lose their ability to adhere to tissue

and low concentration alginate liquids have relatively lower ability to reduce acid diffusion. However, a low concentration may be sufficient at a cellular level to protect against the damage caused by acid and pepsin to cells.

The Caco-2 cell line can not survive in acid when pH value is less than 3. Pepsin is active at pH values lower than 3 (Tobey et al, 2001) thus the effect of pepsin was not examined within this study.

In summary Caco-2 cells were not an ideal cell line for this research. The conflict between the two situations the viscous alginate liquid need longer centrifuge and the longer centrifuge affecting cell viability meant that this study was not successful.

CHAPTER 6 NEW TECHNIQUE ASSESSING OESOPHAGEAL EPITHELIUM DAMAGE AND PROTECTION

6.1 INTRODUCTION

Stratified squamous epithelium on the oesophageal surface is the main barrier to retard acid diffusion into the deeper tissue. Previous work (Batchelor et al, 2002) and the work presented in Chapter 2 suggested that solutions of sodium alginate adhere to oesophageal mucosal for up to 30 minutes and the work described in Chapter 4 demonstrated that an adhesive layer on the mucosal surface significantly reduced acid and pepsin diffusion. This study further investigates the extent of acid and pepsin damage to the oesophageal epithelium and the effect of the adhesive alginate layer in protecting the epithelium from these damage factors.

6.2 MATERIALS AND APPARATUS

6.2.1 Materials

Sodium alginate LF120, supplied by FMC Biopolymer; whose physiochemical properties were described in Chapter 1 and 2; was prepared as an aqueous solution at 2% w/v.

Hydrochloric acid 0.1 M (pH 1), 0.01 M (pH 2) and 0.001 M (pH 3) was prepared by dilution of a 5 M HCl solution (Fisher) 50 times, 500 times and 5000 times, respectively. Acidic pepsin solution (0.3 % w/v) was made by dissolving 0.3 g pepsin A (Sigma, UK) in 100 mL HCl 0.1 M.

After cryo-sectioning the tissue sections were stained using Haematoxylin and Eosin staining method (H & E) which is a standard stain for oesophageal tissue that stains basophilic material blue and acidophilic material pink. Eosin Y is the most common used counterstain to alum haematoxylin in H & E staining. It stains satisfactorily from both aqueous and ethanolic solutions. It was used for staining the cytoplasm into varying shades of pink. Mayers Haematoxylin blue solution and Eosin Y (filtered) were bought from Sigma. 1 g Eosin Y was added in 100mL distilled water to make Eosin Y solution 1 % (w/v). Acid Alcohol 1 % was used as fixation of the tissue that was made by mixing 99 mL of 70 % alcohol with 1 mL concentrated hydrochloric acid.

The collection and storage of the porcine oesophagus was described in Chapter 2 section 2.2.1.1. On the day of the experiment, the frozen oesophagus was taken from the freezer and thawed at room temperature or in saline solution (0.9 % w/v).

6.2.2 Apparatus

Polysine™ microscope slides were purchased from Menzel-Glaser, Germany (Fisher). These slides are coated with an advanced, permanent adhesive to supply superior adhesion to tissue and cell sections that helps to retain the tissue sections on the slide during the process of staining.

The sections were viewed under the microscope (Zeiss Axioskop) and the images were transferred to a computer through a digital camera (Zeiss Axiocam HRC) that connects both microscope and computer to supply digital images for analysis. Imaging software (AxioVision 3.1) was used for image manipulation.

6.3 METHODS

In this study the damage caused by acid and acidic pepsin to oesophageal epithelium and the protection provided by an adhered alginate solution was visualised. Images of oesophageal tissue exposed to acid and acidified pepsin in both the presence and absence of an alginate layer were collected and scored to assess the damage observed.

6.3.1 Preparation of the porcine oesophageal epithelium

The collection, storage and thawing of the porcine oesophagus was described in detail in Chapter 2. The upper epithelial layer of the oesophagus was removed from the tissue section according to the procedure outlined below.

The oesophagus was cut to 2.5 cm length sections and immersed in saline solution at 60 °C for 60 seconds. Upon removal from the saline solutions the oesophageal tissue was placed onto a hot surface to enable easy removal of the upper epithelial layer. Tweezers with sharp tips were used to separate the epithelium layer, the sharp tips were inserted to separate the layers then they could be peeled apart to yield large areas of upper epithelial tissue. Then the epithelium was kept in saline solution until required.

The oesophagus was divided into 3 parts of the top, middle and bottom, the middle part of oesophageal epithelium was used in the experiment. One reason is because the top and bottom parts were quite difficult to isolate from the sub-epithelium tissue. The second reason is to reduce the variation by using the same part of the epithelium.

6.3.2 Diffusion procedure and the designated damage factors

An upper epithelial section of 2 x 2 cm^2 size was cut and put on the bottom of a transwell plate, the section was spread flat and the surrounding tissue stuck to the walls of the well. A volume of 0.04 mL alginate solution (LF120 2 % w/v) was applied on the epithelial surface, left to spread flat and cover the whole epithelium surface. 2.5 mL damage solution (acid or acidic pepsin) was dispersed onto the top of this alginate layer. To test the alginate protective effect, each epithelial tissue was tested against a matched pair for each damage factor, one without 0.04 mL alginate applied, the other one with alginate applied. Different damage factors were described in following paragraphs and listed in tables 6.1 and 6.2.

Acid and pepsin are two factors that cause the damage to the oesophageal mucosal tissue surface (Namiot et al., 1994; Tobey et al., 1989). In order to compare the damage of different concentrations of acid to the oesophageal epithelium, hydrochloric acid solution were used in the experiment at concentrations 0.1 M (pH 1), 0.01 M (pH 2), and 0.001 M (pH 3). Tobey et al (2001) and Furukawa et al (1997) suggested that at the same pH acidified pepsin increased the rate and degree of oesophageal cell and tissue damage. So pepsin solution 0.1 % (w/v), in the gastric contents was used in comparison with the acid alone. 0.1 g pepsin was dissolved in 100 mL of the three concentrations of hydrochloric acid solutions (0.1 M, 0.01 M, and 0.001 M). Table 6.1

lists the factors examined within this study. Initial studies showed that there was little damage to the tissue at pH 3, thus studies at this pH were limited.

Table 6.1 Paired porcine oesophageal epithelial sections with or without alginate coating attacked by hydrochloride acid or acidic pepsin solution at different pH

pH	Acid alone		Acidified Pepsin 0.1 % (w/v)	
1	With LF120 2 %	No LF120	With LF120 2 %	No LF120
2	With LF120 2 %	No LF120	With LF120 2 %	No LF120
3	N/A	No LF120	N/A	No LF120

It was assumed that increased concentrations of acidic pepsin solutions may have the trend to increase damage to the mucosal tissue. Additional studies using increasing concentrations of pepsin (0.1, 0.2 and 0.3 % w/v) at pH 1 were performed in the same manner as those listed in table 6.1. This experimental plan is listed in Table 6.2.

Table 6.2 Paired sections with and without alginate coating attacked by different concentrations of pepsin dissolved in hydrochloric acid pH 1

Pepsin (w/v)	pH1 HCl acid	
0.1 %	With LF120 2 %	No LF120
0.2 %	With LF120 2 %	No LF120
0.3 %	N/A	No LF120

The duration of exposure was another factor tested in the study. It was expected that the longer the duration of exposure the more damage to the oesophageal epithelium. All experiments listed in tables 6.1 and 6.2 were performed with 30 and 60 minute exposures.

6.3.3 Freezing the epithelium

After exposure to acid or acidified pepsin the epithelium was carefully spread onto a wooden board, trying not to damage the tissue surface, a 0.5 x 0.5 cm^2 block was cut and frozen with a liquid nitrogen freezing spray (Chemtronics®). Whilst frozen the block was fixed by embedding it in a drop of embedding medium (Gurr®, BDH) on a cork plate. The freezing spray was used to freeze the sample block within the embedding media prior to storage at –20 °C. The whole operation was performed quickly and the freezing spray was continuous in order to keep the sample frozen. The epithelium sample was very thin and could thaw very easily, which greatly affects the epithelium surface structure that could seriously affect the results.

6.3.4 Sectioning and H & E staining

The temperature of the cryo-section machine (Bright OTF Cryostat) was adjusted to -5 °C for at least one hour prior to taking sections to allow the temperature to equilibrate. All of the samples for sectioning were placed inside the machine. One sample was put on the sectioning platform, the cutting depth of the section was set at 30 μm. The first several sections of the sample cut were wiped away as the edge tissue maybe damaged by the freezing procedure, the next few sections were left on the top of the knife for collection. A slide at room temperature was taken towards the samples, gentle contact was made and the sections adhered to the slide as single flat units. Four or five sections were collected onto each slide that was kept for the staining procedure.

Staining Procedure

The frame holding the slides was gently immersed in Haematoxylin for 2 minutes, then washed with tap water for 10 minutes until the blue colour on the slide was washed away. Then the sections were immersed in 1 % acid alcohol for about 15 seconds and washed with tap water for 10 minutes. Finally the sections were put in Eosin Y solution for 2 minutes and washed with tap water for 10 minutes to remove the excess dye. The coated slides ensured that the sections were retained on the slide surface throughout the staining procedure. After the sections were dried at room temperature, they were taken to view under the microscope. The images were viewed and pictures were taken using a digital camera that connected the microscope to a computer. The images were transferred to the computer and were handled by the image software (AxioVision 3.1) that controlled the light and the clarity of images, several representative pictures were taken and saved for analysis.

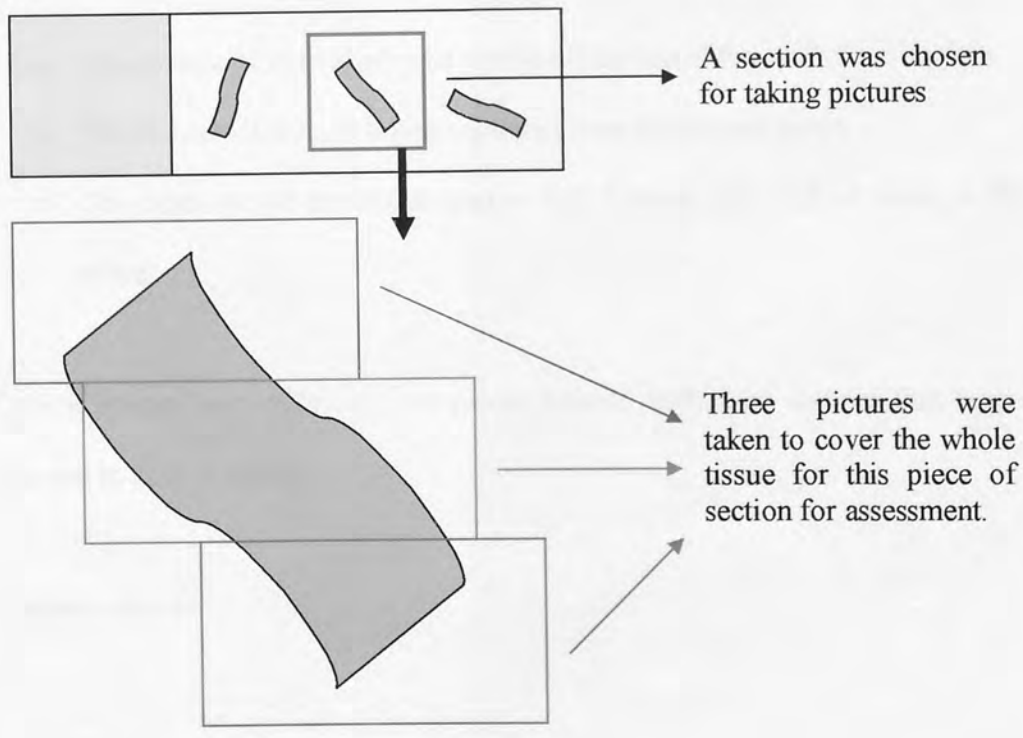


Figure 6.1 Schematic representation of the pictures taken for each section

10 times magnification of the microscopy was chosen to view the images. Occasional damage to the sections including wrinkling or folding of the sections meant that these sections could not be viewed. Sections that were clear and smooth were selected to capture images. As one section could not be taken in total within one picture, several pictures were taken for each section, this is schematically presented in Figure 6.1.

As the epithelium was damaged by acid or acidic pepsin solution at different concentrations, the damage levels were different. In order to assess the damage, an assessment score system (score 0, 2, 4, 6, 8) was set up.

6.3.5 Image assessment criteria

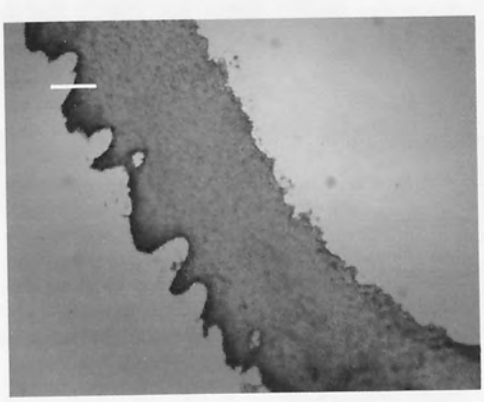
In the assessment criteria, the higher the score, the more serious the damage. There were three aspects set as the assessment criteria.

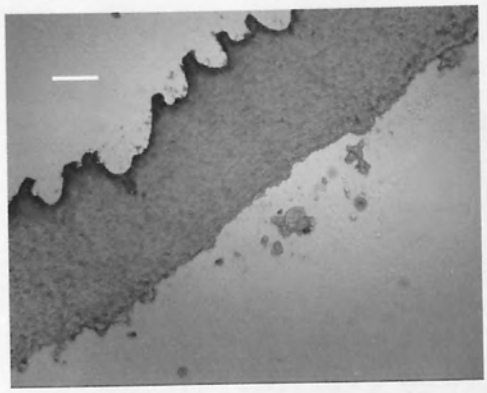
a. The amount of individual cells peeled off the top of the epithelium surface
b. The amount of the cell layers separated from the normal tissue
c. The depth of the tissue damaged, < 1/5, 4 score; 1/5 - 2/5, 6 score, > 2/5, 8 score.

Control images were collected from paired isolated epithelium sections that were not exposed to acid or pepsin.

Control – score 0

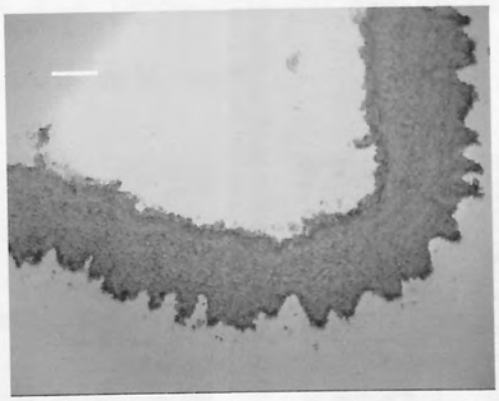
Appearance: the surface was comparably smooth, no surface cell layer damage and individual cells peeled off, no difference between the surface cell layers and the rest of the cell layers in the tissue section.





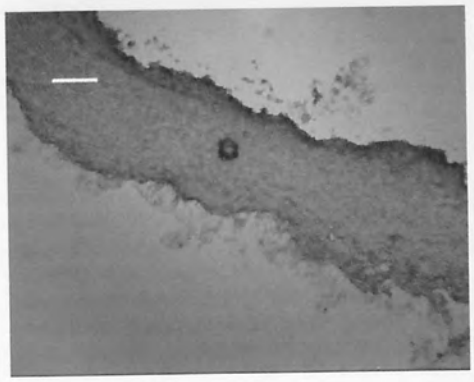
Slightly damage – score 2

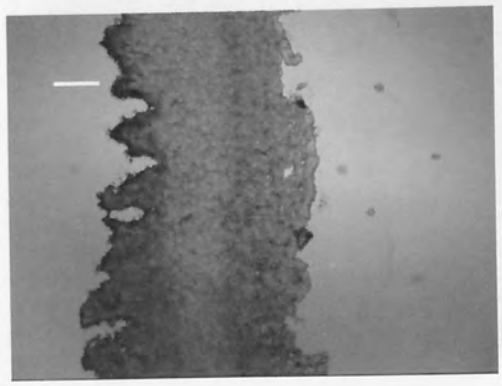
Appearance: surface tissue was slightly damaged, some cells separated on the top of the normal tissue. Few cell layers peeled off, no deep tissue damage.



Moderate damage – score 4

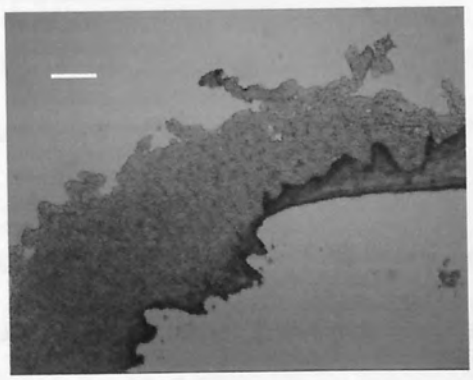
Appearance: surface tissue was damaged visibly, many cells suspended and separated from normal tissue. Some cell layers peeled off from normal tissue. Less than 1/5 depth tissue appeared loose.

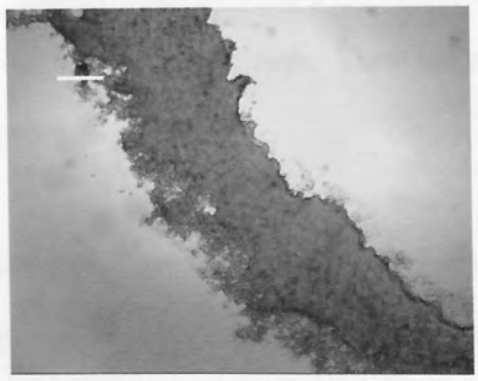




Substantial damage – score 6

Appearance: surface tissue was damaged seriously, many cells suspended and separated from normal tissue. Several cell layers were peeled off normal tissue. 1/5 - 2/5 depth tissue damaged, appearing loose.





Extensive damage – score 8

Appearance: surface tissue was damaged very seriously, a great deal of cells suspended and separated from normal tissue. Deep cell layers were peeled off normal tissue. More than 2/5 depth tissue damaged, appearing loose.

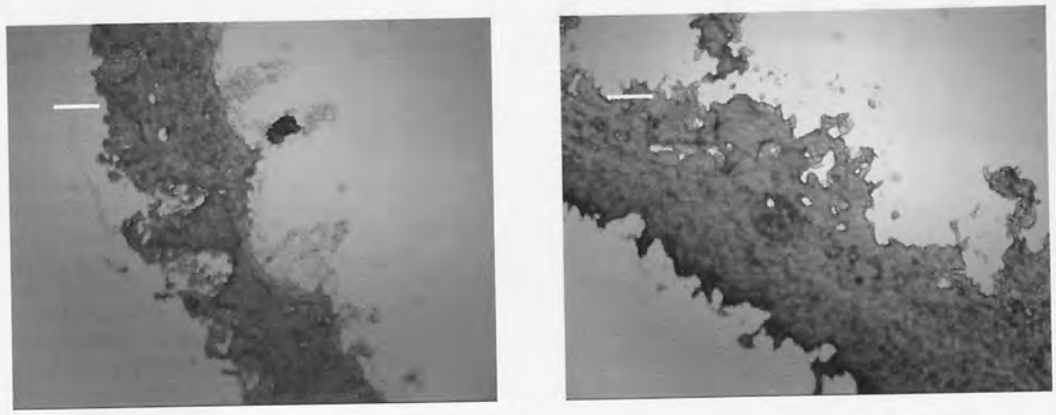


Figure 6.2 Picture examples of the assessment score criteria (calibration bar represents 100 micrometers)

In order to keep the assessment objective, all the pictures were mixed together and edited into two number systems (Table 6.3 and Table 6.4). All of the pictures under the different damage conditions were listed with number and labelled with the experimental condition. The pictures in the other system have only the numbers matched with the first system without labels of experimental conditions. These images were used for volunteer assessors to give scores for each picture.

All of the pictures were listed in a folder for volunteer assessing and scoring presented in Figure 6.3. After reading the score assessment criteria (as described above) the assessors would independently assess each picture and give a score. Part of the score table is shown in Table 6.3 for the volunteer assessors to fill in.

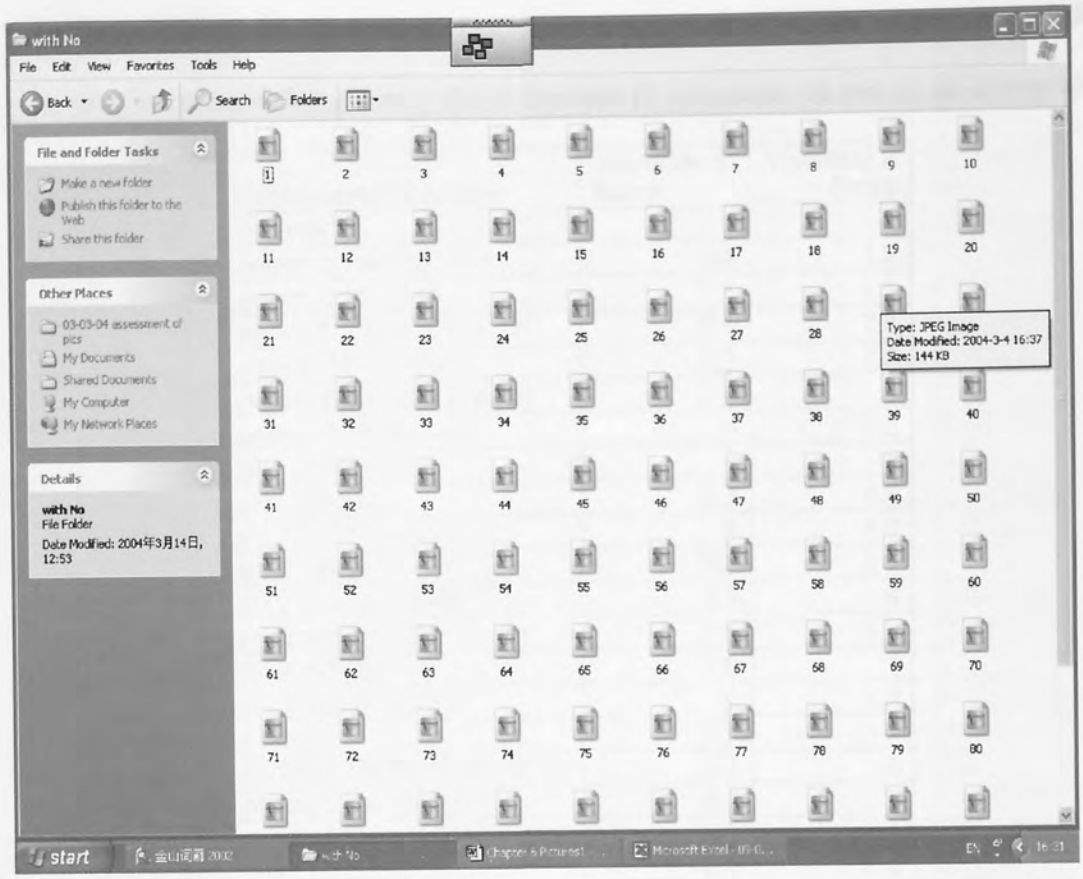


Figure 6.3 Pictures presented to volunteer assessors with number listed

Table 6.3 Part of score table of the pictures for assessor to fill in

pic No.	scores		pic No.	scores	
1			54		
2			55		
3			56		
4			57		
5			58		
6			59		
7			60		
8			61		
9			62		
10			63		
11			64		
12			65		**Name**
13			66		**Date**

Table 6.4 List of the experimental conditions matched with picture number and the score of each condition given by the volunteers (2 volunteers shown as an example)

Experimental situations	Volunteer 1 Score	Volunteer 2 Score
0.5 hour		
pepsin 0.1% lf120 (1-5)	0.8	1.2
pepsin 0.1% (6-9)	5	4.5
pepsin 0.2% lf120 (10-13)	0.5	2.5
pepsin 0.2% (14-16)	5.3	7.3
pepsin 0.3% lf120 (17-20)	0	0
pepsin 0.3% (21-24)	6	5.5
pH1 lf120 (25-28)	0	1
pH1 (29-31)	5.3	4.6
pH 2 lf120 (33-35)	0	4.6
pH 2 (36-38)	3.3	6.6
pH 2 pepsin (43-46)	3.3	4
pH 2 pepsin lf120 (39-42)	0.5	1.5
pH 3 (47-49)	1.3	2
pH 3 pepsin (50-52)	0	1.3
1 hour		
pepsin 0.1% lf120 (91-93)	0.6	2
pepsin 0.1% (94-97)	4.5	3
pepsin 0.2% lf120 (57-59)	2	4
pepsin 0.2% (53-56)	5.3	7.3
pepsin 0.3% lf120 (98-101)	0.5	0
pepsin 0.3% (102-104)	8	8
pH1 lf120 (60.62.64)	1.3	4
pH1 (64.65.67-69)	5.6	6.8
pH 2 lf120 (70-71)	1.3	2
pH 2 (74-76)	2.7	4
pH 2 pepsin (81-83)	3.3	5.3
pH 2 pepsin lf120(77-80)	1	3.5
pH 3 (84-86)	0	2.6
pH 3 pepsin (87-90)	1	2.5

Because of the different length of sections collected on the microscope slide, three to five pictures were taken of each section. So the score of each section was calculated as the average score of the pictures from the same section. As shown in Table 6.4, in the third row pictures No 1 to 5 were under the damage condition of acidic pepsin 0.1 % (w/v) with LF120 2 % (w/v) coated on the epithelium. So the score of this condition was average score of the five pictures. The picture scores given by the volunteers were calculated and listed in table 6.4.

6.4 RESULTS AND DISCUSSIONS

Due to the large number of experimental conditions, this section is divided into subsections of conditions of comparable pH; comparable pepsin concentrations with the same acid solution (pH 1); different pH with the same pepsin concentration (0.1 %, w/v); comparison of coated with alginate sections and without coating.

Table 6.5 Scores of sections under the conditions of different pH acid solution (duration of 0.5 hour and 1 hour, mean ± s.d., n=10)

	Scores	
pH value	0.5 hour	1 hour
1	3.66 ± 1.39	5.54 ± 0.96
2	***5.52 + 1.37***	2.60 ± 1.17
3	1.99 ± 1.59	1.53 ± 0.88

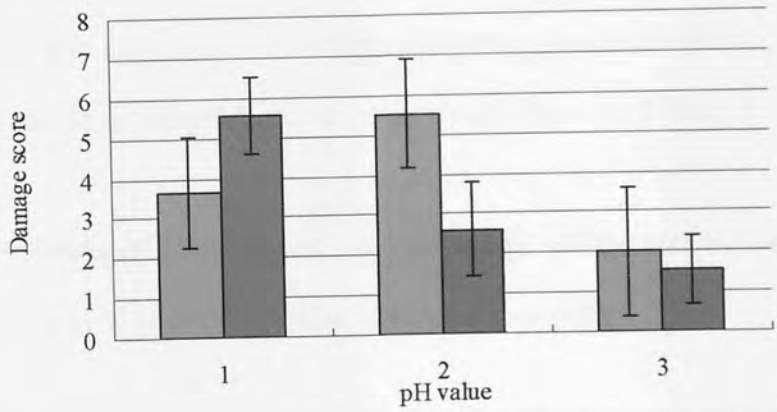


Figure 6.4 Graphic representation of section scores under the conditions of different pH acid solutions (mean ± s.d., n=10, ■ 1hour, ■ 0.5 hour)

For duration comparison (0.5 hr vs. 1 hr), Figure 6.4 shows that at pH 1 the score of one hour duration of acid damage is higher than that of 0.5 hour duration suggesting

that the damage at 1 hour was more than 0.5 hour, however, this difference is not significant ($p>0.05$, ANOVA). At pH 3 both scores are similar demonstrating that the longer time of tissue damage by pH 3 acid did not increase the damage effect. The low score means the epithelium was just a little damaged. At pH 2, the score at 0.5 hour was higher than at 1 hour, which may be caused by tissue variance.

For pH comparison (pH 1, 2, 3 at 1 hour), the sections' scores increased as pH decreased indicating that high concentrations of acid damage the tissue more than low concentrations of acid, as expected. The score at pH 1 is significantly higher than the other two pH values. For 0.5 hour, no significant difference in the scores was determined at each of the concentrations.

Tobey et al (2001) stated that the pepsin increased the degree of oesophageal cell and tissue damage at acidic pH. The study was designed to assess the tissue damage with acidic pepsin and acid alone. The scores of the sections are listed in Table 6.6. Another experiment was designed for increasing the pepsin concentrations (0.1 %, 0.2 %, 0.3 % w/v) in acid solution pH 1 and the score result is listed in Table 6.7.

Table 6.6 Scores of sections under the conditions of different pH with and without pepsin 0.1 % (w/v) (1 hour duration, mean ± s.d., n = 10)

pH value	Scores	Pepsin in HCl	Scores
pH1	5.54 ± 0.96	pH1 pepsin 0.1 %	3.70 ± 0.75
pH 2	2.60 ± 1.17	pH 2 pepsin 0.1 %	3.33 ± 0.95
pH 3	1.53 ± 0.88	pH 3 pepsin 0.1 %	1.55 ± 0.90

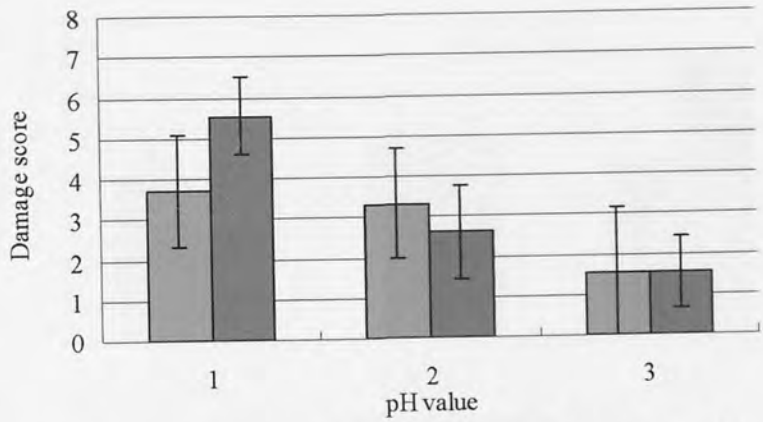


Figure 6.5 Graphic representation of section scores under the conditions of acid and acidic pepsin (mean ± s.d., n=10, ■ acid, ■ acidic pepsin 0.1 % w/v)

According to the theory of Tobey et al (2001), the damage caused by acidic pepsin should be more serious than that caused by acid alone. However, this study demonstrates that the damage scores of the sections exposed to acidic pepsin and acid alone show no significant difference at all three pHs. At pH 1 acid alone damaged the tissue more seriously than acidic pepsin, the scores are 5.54 $\pm$ 0.96 (acid pH 1) and 3.70 $\pm$ 0.75 (acidic pepsin) yet this difference was not significant ($P>0.05$). At pH 2, acidic pepsin scored 2.60 $\pm$ 1.17 lower than acid, which scored 3.33 $\pm$ 0.95, again no significant difference. At pH 3 the scores were quite similar, 1.53 $\pm$ 0.88 for acid alone and 1.55 $\pm$ 0.90 for acidic pepsin.

Table 6.7 Scores of sections under the conditions of different concentrations of pepsin solution (0.1 %, 0.2 %, 0.3 % w/v) in acid solution pH1 (Duration of 0.5 vs. 1 hour, mean ± s.d., n=10).

pepsin conc (w/v)	0.5 hour	1 hour
pepsin 0.1%	4.70 ± 0.59	3.70 ± 0.75
pepsin 0.2%	5.80 ± 1.17	5.74 ± 0.95
pepsin 0.3%	5.00 ± 0.62	7.73 ± 0.46

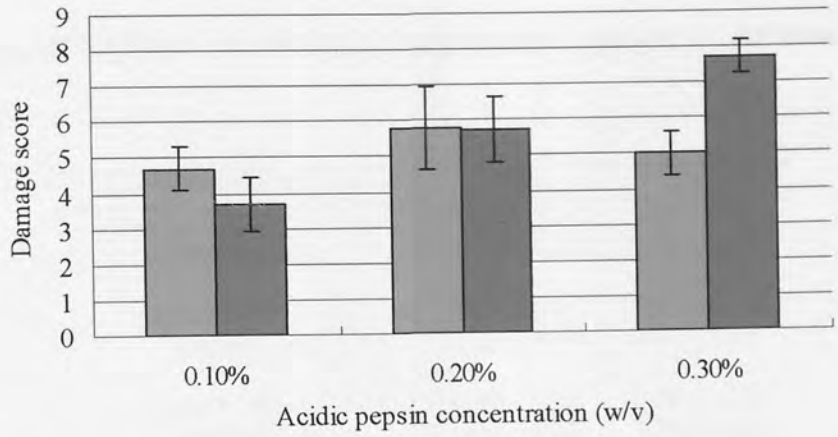


Figure 6.6 Graphic representation of section scores under the conditions of different concentrations of pepsin solution (0.1 %, 0.2 %, 0.3 % w/v) in acid solution pH 1 (Duration of 0.5 vs. 1 hour, mean ± s.d., n=10)

For exposures of 1 hour, the scores of the three pepsin solutions at 0.1, 0.2 and 0.3 % (w/v) showed significant differences with each other ($P<0.05$), which means that increasing concentration of pepsin solutions lead to a significant increase in the level of tissue damage. But for the duration of 0.5 hour, the results did not show a significant difference with increasing concentration. For each concentration there was only a significant difference in the damage observed with the highest pepsin concentration at 0.3 % w/v between 30 minutes and one hour exposure times.

To compare the protective effect of alginate (LF120 2 %) applied to the tissue from the acid and pepsin damage, all the paired scores are listed in table 6.8 for comparison and plotted it in Figure 6.7 (0.5 hour duration) and Figure 6.8 (1 hour duration).

Table 6.8 Scores of sections under all experimental conditions: coated with LF120 and uncoated; different concentrations of pepsin in acid pH 1; different pH acid solutions

Eexperimental conditions	0.5hr	1hr
pepsin 0.1% in LF120	1.08 ± 0.50	1.25 ± 0.80
pepsin 0.1%	4.70 ± 0.59	3.70 ± 0.75
pepsin 0.2% LF120	2.10 ± 0.91	2.81 ± 0.88
pepsin 0.2%	5.80 ± 1.17	5.74 ± 0.95
pepsin 0.3% LF120	0.90 ± 1.26	0.50 ± 0.67
pepsin 0.3%	5.00 ± 0.62	7.73 ± 0.46
pH1 LF120	0.85 ± 1.06	2.71 ± 1.29
pH1	3.66 ± 1.39	5.54 ± 0.96
pH 2 LF120	2.79 ± 2.19	0.60 ± 0.86
pH 2	5.52 ± 1.37	2.60 ± 1.17
pH 2 pepsin LF120	1.75 ± 1.21	2.21± 0.78
pH 2 pepsin	2.73 ± 1.07	3.33 ± 0.95
pH 3	1.99 ± 1.59	1.53 ± 0.88
pH 3 pepsin	1.33 ± 1.12	1.55 ± 0.90

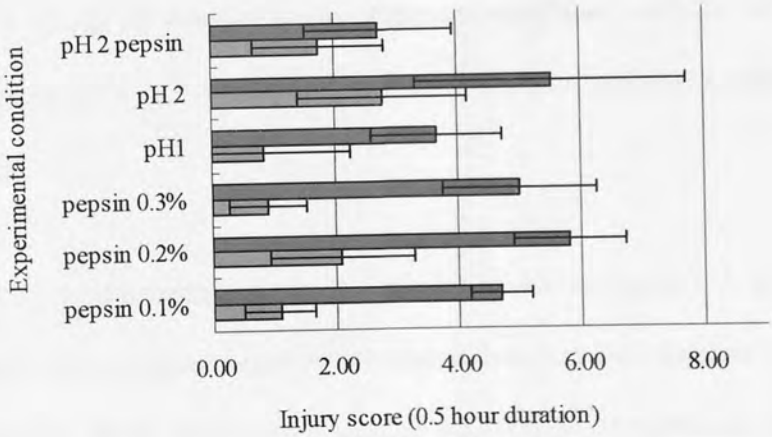


Figure 6.7 Scores of damage under different conditions with or without alginate coating for duration of 0.5 hour (mean ± s.d., n=10, ■ no alginate coating, ■ LF120 2 % coating)

In Figure 6.7 (0.5 hour duration) all the scores of the sections without alginate coating are higher than that with alginate, and under the conditions of all three acidic pepsin concentrations, the scores are significantly lower in the sections with alginate coating than that without alginate coating demonstrating the protection of the alginate greatly protected the tissue from acidic pepsin attack. The results at pH 2 were higher than expected although not significantly different to those at pH1 or pH 3, this may be due to natural variation in the tissue sections used.

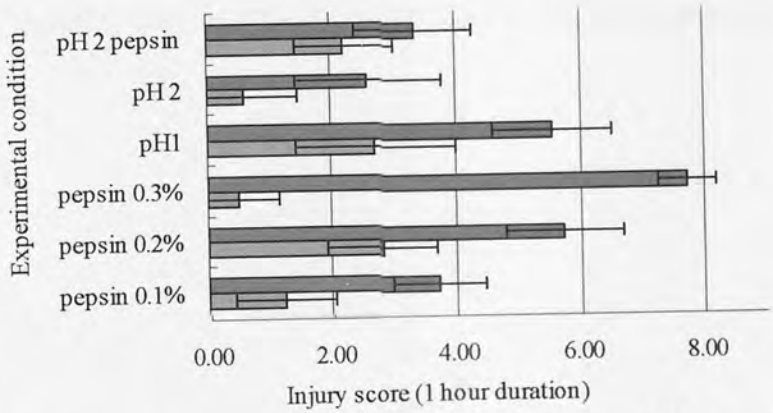
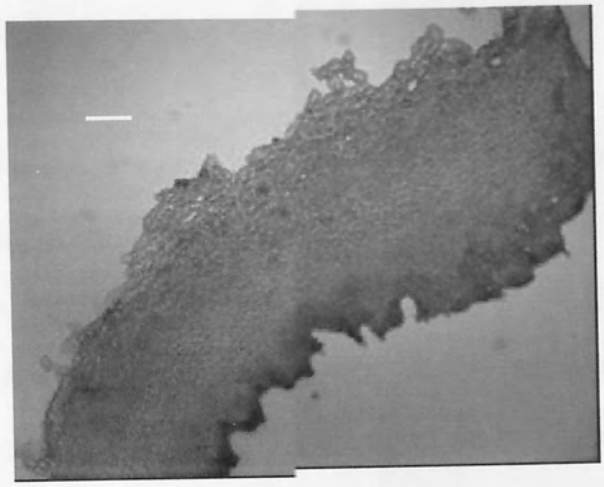


Figure 6.8 Scores of damage under different conditions with or without alginate coating for duration of 1 hour (mean ± s.d., n=10, ■ no alginate coating, ■ LF120 2 % coating)

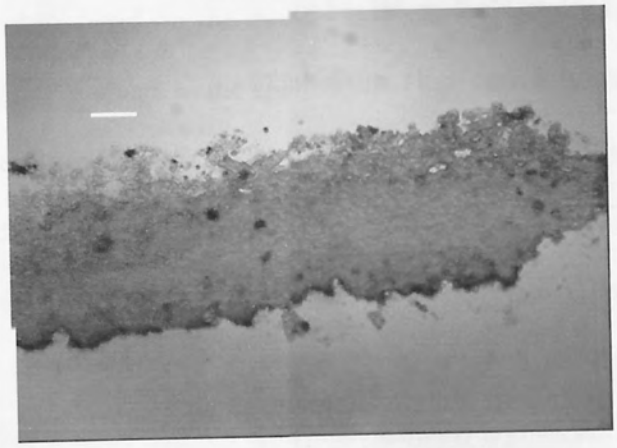
Figure 6.8 (1 hour duration) shows the similar results as Figure 6.7, all the scores of the sections without alginate coating are higher than that with alginate coating. Except at pH 2 pepsin, all the other paired sections scores showed significant difference with each other ($P < 0.05$). So it is suggested that an alginate layer (LF120) can greatly protect the tissue from the damage of acid and pepsin at 1 hour duration.

From observation of the images it was noted that the damage caused by acid alone and acidic pepsin were histologically different (Figure 6.9). The damage caused by acid

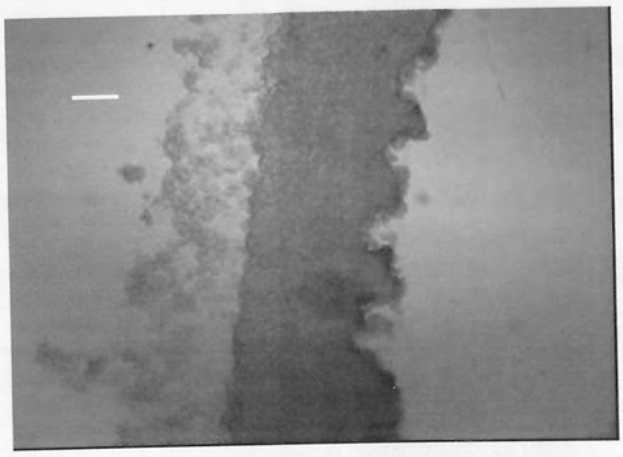
alone in Figure 6.9 (a) showed layer or blocks of cells isolated from the undamaged tissue. But the damage caused by acidic pepsin was different from the acid damage, the damaged cells were cloudy and looked like many detached individual cells clumped together Figure 6.9 (b,c). A study by Tobey et al (2001) indicated that pepsin caused cell destruction with dramatic loss of cells from the upper two thirds of the epithelium, yet little damage was observed using acid alone. The mechanism of this damage is not fully understood although it has been suggested that pepsin affects paracellular rather than transcellular pathways and thus it is damage to the cell junctions caused by exposure to pepsin that leads to the damage observed (Tobey et al. 2001).



a. pH 1 (1 hour)



b. pH1 pepsin 0.3 % (1 hour)



c. pH 1 pepsin 0.1 % (1 hour)

Figure 6.9 Picture comparison of oesophageal epithelium damaged by acid and acidic pepsin (calibration bar represents 100 micrometers)

6. 5 CONCLUSIONS

This chapter describes a new *in vitro* technique to assess the level of damage to oesophageal mucosal epithelium caused by acid and pepsin, the two main components in gastric reflux. The potential of alginate in protection of the tissue from injuries caused by acid and pepsin was also evaluated. A scoring system was set up to assess the damage level of the tissue and the results were statistically analysed.

An adhesive alginate layer on the oesophageal epithelium leads to a reduced score indicative of reduced damage to the epithelium. High concentrations of pepsin at pH 1 damaged the tissue significantly more compared to damage with low concentrations of pepsin (1 hour duration). A lower pH leads to greater damage of oesophageal tissue yet this difference was significant at 1 hour between pH 1 and both pH 2 and 3. Exposure times of 1 hour compared to 30 minutes lead to greater damage in all cases although the effect of time was not always statistically significant.

The individual variability of the oesophageal tissue greatly affected the results, with some tissue sections exhibiting extensive barriers to diffusion of both acid and pepsin. Small tears in the epithelium that were not visible to the naked eye greatly affected results, meaning that many experiments were repeated. Although the scoring criteria was shown and demonstrated to all volunteer assessors, individual judgement played a large role in the score results and lead to large variations.

In summary although there are many shortcomings for this experimental technique, it is very promising in assessing tissue damage and protection.

CHAPTER 7 GENERAL DISCUSSIONS AND CONCLUSIONS

Previous work (Batchelor et al, 2002) has stated that viscous alginate liquid adheres to porcine oesophageal tissue for up to 60 minutes, this has a function as a protective layer against gastric reflux. In this study, the bioadhesive properties of alginates were investigated and the function of alginate as a protective layer adhering to the oesophagus was studied by different methods.

7.1 THE RETENTION STUDY

The retention study investigated aqueous alginate retention on the oesophageal surface and the influences of many factors on the retention including; alginate type; concentration; aggressive factors in gastric contents, like acid and pepsin; and the potential of the aqueous alginates as a drug delivery system. The experimental results confirmed that higher molecular weight liquid alginates were retained for longer periods on porcine oesophageal tissue compared to alginates with lower molecular weights. Increasing the concentration of liquid alginate improved the retention. Lower pH buffer increased the retention but pepsin as washing media decreased the retention.

LFR5/60 was retained to the least extent compared to LF120 and H120 due to its low molecular weight and therefore reduced viscosity at the same concentrations as the other alginates. However, this low inherent viscosity meant that higher concentrations

of LFR5/60 are still pourable and can minimise the diffusion of acid and pepsin. In addition, the high G content of this alginate made it the best at reducing the diffusion of both acid and pepsin due to the tight mesh formed by hydrogen bonding between the G blocks. Therefore a higher molecular weight, high G alginate may be the best choice as an oesophageal protective bioadhesive.

In the diffusion study although commercial formulations presented very strong capacity to reduce acid diffusion at set depth 0.44 mm, they showed little retention on the oesophageal tissue. Their ability to reduce acid diffusion was greatly weakened without certain depth of these formulations coating on oesophageal mucosa.

7.2 THE VISCOSITY STUDY

Table 7.1 Relationship between viscosity and retention of liquid alginates

	Viscosity (cp)	Retention (% of alginate retained after 30 minutes)
↑ Molecular weight	↑	↑
↑ Concentrations of alginate	↑	↑
↑ Hydrogen concentration	↑	↑
↑Presence of gastric mucin	↑	N/A
↑ Presence of gastric pepsin	↓	↓

The viscosity study measured the viscosity of liquid alginates under the influence of difference factors (pH; concentration; presence of pepsin; presence of mucin; duration of time). Data showed that lower pH, higher concentration of alginates and the presence of mucin all increased the viscosity, but the presence of gastric pepsin decreased the viscosity of liquid alginates. In addition, the viscosity of an alginate

solution was observed to decrease over time. A relationship between the retention and viscosity was found and is shown in Table 7.1.

The trend in Table 7.1 shows that as viscosity increased with certain factors, the retention was also increased. But there was no linear relationship between them. For example the viscosity versus retention is shown in table 7.2 for LF120 at a range of concentrations yet no linear relationship is observed.

Table 7.2 Viscosity and retention of LF120 affected by different concentrations (mean ± s.d.)

Concentration of LF120 (w/v)	Viscosity (cp)	Retention (% of retained after 30 minutes)
1 %	20 ± 0	2.15 ± 4.3
2 %	1147 ± 60	16.88 ± 1.45
4 %	143000 ± 1692	26.81 ± 5.35

Doubling the concentration increased the viscosity of the alginate (LF120) by a factor of two on a log scale, but the retention just slightly was increased. Although pepsin decreased both the viscosity and the retention of the alginate again no linear relationship was determined, as shown in table 7.3.

Table 7.3 Viscosity and retention of LF120 2 % (w/v) affected by pepsin 0.1 % (w/v) (mean ± s.d.)

Pepsin concentration in LF120 (w/v)	Viscosity (cp)	Retention (% of retained after 30 minutes)
0 %	1413 ± 220	16.9 ± 1.5
0.1 %	1240 ± 245	0 ± 1.9

In this case addition of pepsin just slightly decreased the viscosity by about 200 cp, but the retention was greatly decreased to 0 % after 30 minutes. Both of the above examples indicated that the viscosity is not the only parameter to affect the retention. Different factors have their own mechanisms to influence the retention of alginate on tissue. For example, molecular chain entangling can affect the intermolecular forces, and physically (or chemically) change the molecular structure. More studies need to be performed to investigate these mechanisms.

7.3 THE DIFFUSION STUDY

The diffusion study demonstrated the capacity of an alginate layer to protect the tissue from damage caused by gastric refluxate. The data showed that the alginate liquid layer can strengthen the pre-epithelial defensive mechanisms by retarding the acid and pepsin diffusion thus decreasing the contact of acid and pepsin with oesophageal apical cells.

A mathematical function was introduced to connect the retention data and the diffusion data from the studies performed. In the retention experiments the percentage of the sodium alginate retained on tissue reduced over time (chapter 2 Retention study). In the acid diffusion experiments the rate of acid diffusion decreased with increasing depth of alginate layer applied (chapter 4 Diffusion study). So a function was set up using the retention data to link the percentage of retained alginate and time, another linear relationship between percentage of acid diffusion and the depth of applied alginate layer was found from the diffusion data, then the relation between the percentage of acid diffusion and the time was set up.

An example of this mathematical function is given below using LF120 2 % retention on oesophageal tissue as the model. Figure 7.1 shows the retention graph.

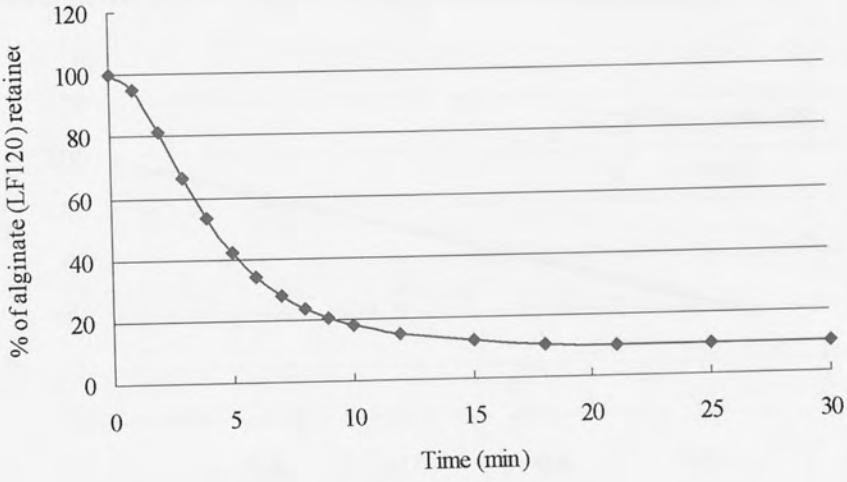


Figure 7.1 Retention of alginate LF120 (2 %, w/v) over time

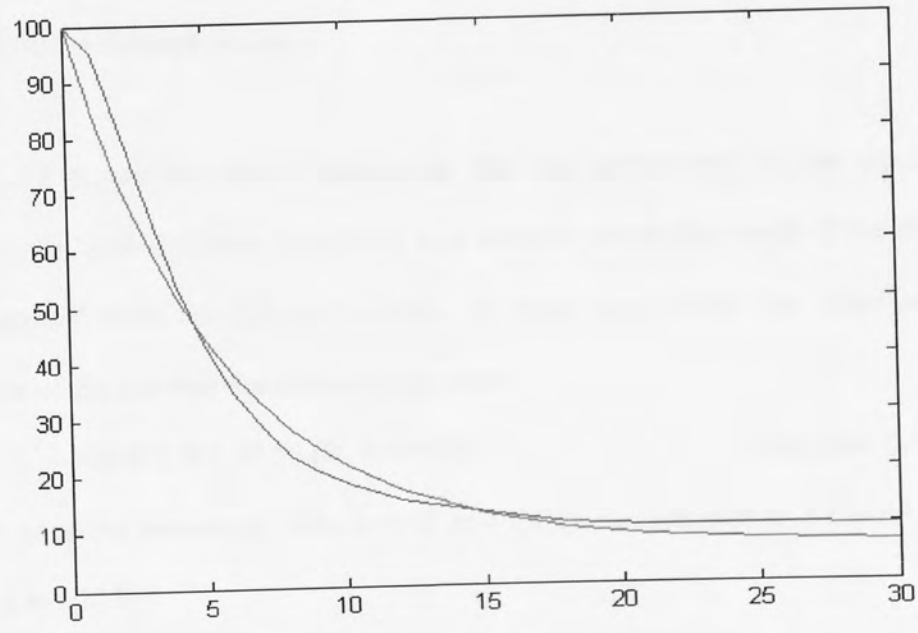


Figure 7.2 Matlab software set up a fittest function for alginate LF120 (2 %, w/v) retention (blue line – real retention curve, red line – best fit function curve)

Figure 7.2 shows this retention data with a line of best fit that was calculated using Mathlab software. The equation of the line of best fit (red line) is

$$y\ \% = 6.4 + 93.6\ e^{-(x/5.45)}. \qquad \textit{Equation 7.1}$$

Where y is the percentage of applied alginate retained and x is time.

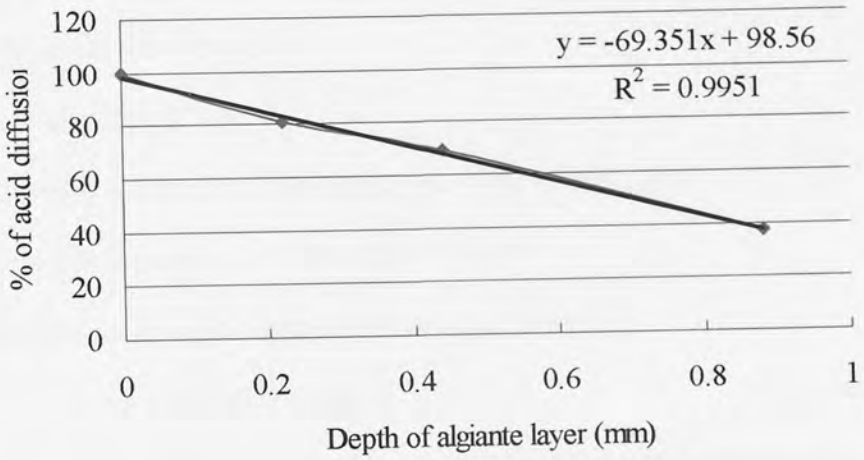


Figure 7.3 Relationship between the depth of alginate layer and the percentage of acid diffusion through the layer

Figure 7.3 shows the linear relationship that was determined for the percentage reduction in acid diffusion (compared to a control) versus the depth of the alginate layer applied from the diffusion studies. A linear relationship was found and the equation of the line was determined using excel;

$$y' = -69.351\ D + 98.56\ (R^2 = 0.9951). \qquad \textit{Equation 7.2}$$

Where y' is the percentage reduction of acid diffusion compared to a control which was set as 100 %

D = the depth of the applied alginate layer

The link between the two equations is the depth of the liquid alginate retained on tissue that could be expressed at set time points in the retention experiment;

The original volume applied was 1 mL (1000 mm^3) and the tissue surface area was;

$1.5 * 8 = 12\ cm^2 = 1200\ mm^2$;

Therefore the original depth is the volume divided by the surface area, and the percentage retained can be used to calculate the percentage of the original depth present on the tissue surface.

Original depth = (1000/1200)

The depth as a function of the percentage = y%*(1000/1200)

So the depth D (mm) = y %*1000/1200 = y/120.

So this form of D can be introduced into equation 7.2:

$$y' = -69.351\ (y/120) + 98.56 \qquad \textit{Equation 7.3}$$

The y in equation 7.3 is that from equation 7.1 and this can be rewritten in terms of x:

$$y' = -69.351\ ((6.4 + 93.6\ e^{-(x/5.45)})/120) + 98.56$$

And this can be further simplified:

$$y' = 94.86 - 54.09\ e^{-(x/5.45)}$$

This equation now directly relates the level of protection offered against acid diffusion (y') as a function of retention time (x).

This result can be tested using some example times:

set t = 5.45 minutes (as this is an easy function to work with), y' = 74.96 that means at 5.45 minutes, the liquid alginate layer retained on tissue reduces acid diffusion by 26 %. set t =10.9 minutes, y' = 87.54 that means at 10.9 minute, the liquid alginate layer retained on tissue reduces acid diffusion by nearly 12 %.

However this calculation assumes that the liquid alginate is evenly distributed on the tissue surface over time, which may not be a realistic assumption.

7.4 CELL CULTURE EXPERIMENTS

This idea assumed that alginate liquid can adhere to individual cells to protect the cells from acid and pepsin. But, unfortunately this study did not provide usable data however, there were some promising results that showed the possibility of liquid alginate in protecting individual cells against acid injury.

7.5 TISSUE DAMAGE STUDY

Chapter 6 used a microscopy-based technique and a score system to assess the oesophageal epithelial damage caused by aggressive factors (pepsin and acid), and compared the injuries from alginate coated tissue and uncoated tissue. The results demonstrated the effective protective function of the liquid alginate coating on epithelium against the damage caused by pepsin and acid.

Heartburn and acid regurgitation are considered the primary symptoms of GORD. A prevalence survey showed that the percentage of the population with these symptoms yearly in western countries including the UK were 29 – 40 %, and even higher in US, up to 58.7 % (Locke et al, 1997); eastern countries have a relatively lower percentage; such as China, 9 – 16.9 % reporting the symptoms, and 6 % people in Singapore (Kang & Ho, 1999). The prevalence of GORD is still rising, a survey showed that in 1970s only 3 % Japanese people were diagnosed as reflux oesophagitis; it jumped to 10 – 15 % in the late 1990s (Hongo & Shoji, 2003). Some complications of GORD like dysphagia, oesophageal strictures greatly damage life quality. GORD increases

the likelihood of developing Barrett's Oesophagus and oesophageal carcinoma, which can threaten people's lives. Therefore effective treatment of GORD and control of the symptoms are very important in therapy.

Generally drinks and food take 7 -10 seconds to transit through the oesophagus to the stomach. Such a short transit time makes it very difficult to administer drugs to treat oesophageal diseases locally. Sodium alginate is a natural polysaccharide extracted from seaweed. This study investigated the retention profile of aqueous sodium alginate to porcine oesophageal mucosa and confirmed its ability to adhere to oesophagus and its capacity to retard the diffusion of hydrogen ions and pepsin (two primary components in acidic gastric refluxate), so to reduce the contact of gastric contents with the oesophageal mucosa, furthermore the experimental results also proved the protection of liquid alginate to the oesophageal epithelium from the damage caused by acid and pepsin. In addition, aqueous alginate was tested and demonstrated a very good potential as a drug carrier to increase the drug retention on the oesophageal mucosa.

These research results demonstrate that sodium alginate is an excellent mucoadhesive to adhere to oesophageal mucosa treating Gastro-oesophageal Reflux Disease. Its molecular stability and safety for oral administration make it a very promising candidate polymer to treat gastro-oesophageal reflux disease, reduce people's sufferance from this disease and improve quality of life.

List of peer-reviewed publications

Journal

1. H.K. Batchelor, **M. Tang**, P.W. Dettmar, F.C. Hampson, I.G. Jolliffe, and D.Q.M. Craig, "Feasibility of a bioadhesive drug delivery system targeted to oesophageal tissue," *European Journal of Pharmaceutics and Biopharmaceutics, 57(2004) 295-298*

2. M. Tang, H.K. Batchelor, P.W. Dettmar, "Bioadhesive oesophageal bandages: protection against acid and pepsin injury" submitted to *International Journal of Pharmaceutics*, March 2004

3. M. Tang, H.K. Batchelor, P.W. Dettmar, "A novel in vitro technique to measure damage to oesophageal epithelium caused by acid and acidified pepsin solutions". In preparation, to be submitted to *European Journal of Pharmaceutical Sciences.*

Conference

1. M. Tang, P.W. Dettmar, H.K. Batchelor, "An *in vitro* examination of solutions of sodium alginate as oesophageal protectants against damage caused by gastric reflux" *accepted as poster presentation and podium presentation in BPC (British Pharmaceutical Conference), Manchester, UK, Sept. 2004*

2. K.K. Bains, **M. Tang**, H.K. Batchelor, "Pectins as oesophageal protectants; their bioadhesive potential and ability to reduce acid and pepsin diffusion" *abstract submitted to BPC (British Pharmaceutical Conference), Manchester, UK, Sept. 2004*

3. **M. Tang**, P.W. Dettmar, H.K. Batchelor, "Adhesive solutions of sodium alginate protect the oesophagus against damage caused by gastric reflux; an vitro study" *abstract submitted to the 12th UEGW (United European Gastroenterology Week), Progue.Czech Republic, Sept. 2004*

4. M. Tang, P.W. Dettmar, H.K. Batchelor, "A Bioadhesive alginate layer on the oesophagus can reduce damage caused by acid-reflux" *in Proceedings of PSWC (Pharmaceutical Sciences World Congress), Page 214 (P1E-IV-079) Kyoto, Japan, May 2004*

5. M. Tang, H.K. Batchelor, P.W. Dettmar, I. Jolliffe, F. Hampson, "Bioadhesive Drug Delivery System for Local Targeting of Oesophageal Tissue," *in Proceedings of the 30th Anniversary of the Controlled Release Society, No. 365, Glasgow, UK, Jul. 2003.*

6. M. Tang, H.K. Batehclor, P.W. Dettmar, I. Jolliffe, F. Hampson, "Alginate solutions targeted to the oesophagus as a barrier to reduce the diffusion of hydrogen ions with the gastric content," *Poster presentation at a national conference on Reflux in the Upper Aero-Digestive Tract in Nottingham University, UK, Apr. 2003.*

7. M. Tang, H.K. Batchelor, P.W. Dettmar, F.C. Hampson and I.G. Joliffe, "Bioadhesive drug delivery system for local targeting of oesophageal tissue," *in Proceedings of AAPS (American Association of Pharmaceutical Scientists), Canada, Nov. 2002.*

Appendix: Materials and chemicals used in the project

Materials	Batch No.	Catalogue No.	Supplier
Carbopol 974P			
Dulbecco's modified eagle medium (DMED)			Invitrogen, UK
EDAC		E7750	Sigma-Aldrich, UK
EDTA			Invitrogen, UK
Eosin Y		E-4382	Sigma-Aldrich Chemie Gmbh, Germany
Foetal calf/bovine Serum (FCS)			Invitrogen, UK
Gaviscon Advance®			Reckitt & Benckiser Healthcare, UK
Gaviscon Liquid®			Reckitt & Benckiser Healthcare, UK
Gaviscon Mikstur®			Ferring Legemidler, AS, Norway
Glass microfibre (GF/C) filter paper		FDJ-515-030T	Fisher (whatman®), UK
Gum Xanthan		G-1253	Sigma-Aldrich Chemie Gmbh, Germany
Harris Haematoxylin, solution modified		HHS-16	Sigma Dignostics, USA
Hydrated dialysis membrane		TWT- 400-070M	Fisher Chemical, UK
Latex Beads, polystyrene, chemically modified, fluorescent (yellow-green, 2μm diameter)			
- Carboxylate-modified		L3030	Sigma-Aldrich, UK
- Amine-modified		L0905	Sigma-Aldrich, UK
- Sulfate-modified		L1903	Sigma-Aldrich, UK

Minimum essential medium (MEM)			Invitrogen, UK
Mucin type II: crude		M2378	Sigma-Aldrich, UK
Pepsin A, lyophilized powder		P7012	Sigma-Aldrich, UK
Polysine™ microscope slide			Fisher (Menzel-Glaser®), Germany
Protanal, H120L Protanal, LF120 Protanal, LFR5/60	240304/645209 907788 599345		FMC Biopolymer, Norway
Technetium ($^{99}Tc_m$)			City Hospital, Birmingham
Trypan blue solution		T8154	Sigma-Aldrich, UK
Trypsin			Invitrogen, UK
Disodium Fluoresceinamine		F6377	Sigma-Aldrich, UK
$CaCO_3$		23,921-6	Sigma-Aldrich, UK
HCl		H7020	Sigma-Aldrich, UK
$KHCO_3$		23,720-5	Sigma-Aldrich, UK
K_2HPO_4		P3796	Sigma-Aldrich, UK
NaCl		S9888	Sigma-Aldrich, UK
$NaHCO_3$		S5761	Sigma-Aldrich, UK
NaH_2PO_4		33,198-8	Sigma-Aldrich, UK
NaOH		22,146-5	Sigma-Aldrich, UK
$Al(OH)_3$		A1577	Sigma-Aldrich, UK

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