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NOVEL FORMULATIONS FOR USE IN THE ORAL CAVITY

PRESENTED BY

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DECLARATION

This thesis is submitted by the undersigned to the University of Dublin, Trinity College, for examination for the Degree of Doctor of Philosophy. It has not been submitted for a degree at any other university. It is entirely the work of the author, except where duly acknowledged.

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Helena Kelly

Helena Kelly, B.Sc. (Pharm.), M.P.S.I.

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ABBREVIATIONS AND SYMBOLS

µm	Micrometre
ANOVA	Analysis of variance
AUC	Area under the curve
BNF	British National Formulary
BOP	Bleeding on probing
BP	British Pharmacoepia
CAL	Clinical attachment level
CFU	Colony forming unit
cm	centimetre
Conc.	Concentration
Da	Daltons
DDWMS	Drum dried waxy maize starch
DPGM	Dried porcine gastric mucin
EDTA	Ethylene diamine tetraacetic acid
Eqn.	Equation
F_{max}	Maximum force of detachment
FPGM	Fresh porcine gastric mucin
FPOM	Fresh porcine oral mucosa
g	grams
HAP	Hydroxyapatite
HPLC	High performance liquid chromatography
hr	Hour (s)
l	Litre (s)
M	Molar
MBC	Minimum bactericidal concentration
mg	Milligram (s)
MIC	Minimum inhibitory concentration
min	Minute (s)
ml	Millilitre (s)

mM	Millimolar
mN	Milli Newtons
n	Number of samples in a data set
N	Newtons
ng/ml	Nanograms per millilitre
nm	Nanometre
°C	Degrees celsius
PDF	Peak detachment force
PEG	Polyethylene glycol
pH	$-\log_{10}H^{+}$
PPD	Probing pocket depth
ppm	Parts per million
r	Correlation coefficient
rpm	Revolutions per minute
s	Seconds
SD	Standard deviation
SEM	Scanning electron microscopy
TID	Three times daily
USP	Uniteds States Pharmacoepia
UV	Ultra-violet
v/v	Volume in volume
vs.	versus
w/v	Weight in volume
WOA	Work of adhesion

SUMMARY

The aim of this thesis was to develop a number of novel products for use in the oral cavity. Periodontitis is a condition characterized by the loss of clinical attachment to the gum, along with resorption of the alveolar bone. This process results in the formation of a periodontal pocket, increased tooth mobility and ultimately tooth loss. The condition develops as a result of a localized bacterial infection of mixed pathogenic microflora, due to the accumulation of subgingival plaque. A novel drug delivery system was developed, for the localized treatment of periodontitis. The localized treatment of the condition is considered preferable as high local concentrations can be achieved, without concomitant side effects. It used the novel excipient, halloysite, to offer a controlled release of tetracycline base. Halloysite is a microtubular material and it is possible to load drug into the lumen of the microtubules, after which a chitosan coating can be applied, which further retards release from the halloysite. Tetracycline was the antibiotic chosen for the product, as it has been widely shown to be efficacious against periodontal pathogens. A delivery system based on a thermoresponsive polymer, *poloxamer 407*, was developed, as it offered ease of administration. Both the gelling temperature and the adhesive ability of the polymer system was modified, to produce the most suitable system. The final product was tested *in vivo* in a dog model, and showed good retention and positive antimicrobial activity, with drug release occurring over a 6-week period.

Xerostomia is a condition characterized by a reduction in or loss of saliva production. It can be caused by Sjögrens syndrome, radiation of the head and neck, drug therapy, HIV and diabetes. Saliva has a wide range of functions in the oral cavity and its loss can result in a number of problems, including increased risk of dental caries and *Candida* infections, oral mucositis, difficulty in eating and talking. Current treatments available include pilocarpine, a salivary stimulant and saliva substitutes. Pilocarpine may be unsuitable for certain groups of patients and is associated with numerous side effects. Saliva substitutes offer poor permanence and often have an unsuitable texture for use in the oral cavity. A new formulation was developed and extensive *in vitro* testing performed to evaluate its bioadhesion

and lubricity. The method of testing for bioadhesiveness was also extensively studied.

Fluoride is an important mineral in the prevention of dental caries. Its mechanism of action is complex and not yet fully understood, however it is evident that it reduces the acid solubility of enamel. Fluoride containing preparations are numerous and can be generally divided into those needing professional application, which normally provide a sustained release and those that can be applied by the patient themselves, which generally have a short term effect. Flouride can also be obtained via the water supply, however this method of fluoride application is currently under close scrutiny. A controlled release bioadhesive fluoride tablet was developed, as there is currently a lack of products available that can be patient applied and offer controlled release. Halloysite was once again utilized, in conjunction with a solid lipid microparticle, to improve tabletting and controlled release. A bioadhesive backing layer, containing *Carbopol 974P* was developed. Approximate zero order release of fluoride was observed over an 8 hr period, with the amount of drug released, suitable for the adult daily requirement of fluoride.

Chapter 1

Introduction

1.1 Origin and scope of thesis

The aim of this work was to develop a number of novel products for the treatment of specific oral conditions. The first product to be developed was a local drug delivery system for the treatment of periodontitis. Secondly a product was to be formulated to assist in the alleviation of the symptoms associated with xerostomia. The final product was a fluoride containing bioadhesive tablet to be used in both the prevention of dental caries and in the reversal of early caries lesions.

Periodontitis is a localized inflammation of the periodontal pocket caused by bacterial infection, which can result in loss of clinical attachment, resorption of alveolar bone and ultimately tooth loss. Treatment of this condition with antibiotics in localized delivery systems is becoming more widespread (Goodson *et al.*, 1985, Killoy, 1998, Southard and Godowski, 1998). Products currently available can be difficult to apply to the periodontal pocket and have comparatively short release profiles, usually giving a maximum of a 10-day release. The aim of this work was to develop a product that was easy to administer, had good retention in the periodontal pocket and which offered a more prolonged release of antibiotic. Tetracycline was the antibiotic of choice for this product because it has been shown to be effective against periodontal pathogens (Miyake *et al.*, 1995) and is currently used in a commercially available product for the treatment of periodontitis (Goodson *et al.*, 1991). The tetracycline was loaded into the novel excipient halloysite and coated with the polymer, chitosan, in order to provide a sustained release of the drug. The delivery system for the drug-loaded halloysite was considered to be important, as ease of delivery is a key issue with local drug delivery systems for the periodontal pocket. The delivery system developed consisted of *poloxamer 407*, a thermoresponsive polymer system, with added polyethylene glycol, which altered the thermoresponsivity of the system. This made the system syringeable at room temperature but a thick gel at body temperature. Octyl cyanoacrylate was also incorporated to increase tissue adhesion and therefore improve retention. The efficacy

of this product was examined *in vivo* using a dog model, which examined the drug release and the impact on the microbiology of the periodontal pocket.

Xerostomia is a condition characterized by a reduction or loss in salivary flow often with a concurrent change in the composition of the saliva, resulting in dryness of the oral cavity. This in turn leads to changes in oral pH and oral microflora, resulting in increased risks of infection, dental caries, mucositis, gingivitis and periodontitis, as well as difficulties with swallowing, speaking and mastication. The two primary treatments currently available for xerostomia are the salivary stimulant, pilocarpine and saliva substitutes. Pilocarpine is only useful where there is residual salivary gland activity and it has a poor side effect profile. Salivary substitutes are cheap, safe and easy to use; however current formulations lack permanence, often fail to improve symptoms and are frequently too tacky in the mouth. By taking into consideration the numerous problems facing xerostomia sufferers and trying to address these at formulation level, a preparation was developed which is hoped to be a considerable improvement on those currently available. The adherence of the product in the oral cavity was addressed through the use of the bioadhesive polymer *Carbopol*™ (Smart *et al.*, 1984), while problems with regard to lubricity were addressed through the use of vegetable oils. Extensive *in vitro* testing was performed to characterize and evaluate the product. The rheology, bioadhesion and lubricity of numerous formulations were examined prior to determining a final product. Preliminary *in vivo* testing of two lead formulations indicated that the type of xerostomia suffered by the patient may be important in determining the efficacy of the product.

Dental caries are a problem associated with patients who suffer from xerostomia, but also can occur with several other groups of patients. Orthodontic patients suffer increased risk of caries (Gorelick *et al.*, 1982) as do populations living in areas that do not have fluoride supplementation of their municipal water supplies (O'Mullane *et al.*, 1988). There are currently a large number of formulations available that deliver fluoride supplementation. In general these can be divided into professionally applied and patient

applied. Professional applications often offer sustained release e.g. fluoride varnishes, whereas patient applied formulations offer short-term high concentrations e.g. toothpastes, mouthwashes. The purpose of this section of the project was to develop a patient applied product that offered sustained release properties along with ease of application. Halloysite was once again used to help provide a sustained release of drug. However due to the high water solubility of sodium fluoride, the drug loaded halloysite was entrapped in a solid lipid microparticle to reduce the rate of release of the drug. In order to allow for application of the product in the oral cavity, a bioadhesive bilayered tablet was formulated. The lower layer of this tablet consisted of a bioadhesive layer while the upper layer contained the drug entrapped in the halloysite/solid lipid microparticles.

1.2 Anatomy and physiology of the periodontium

1.2.1 Introduction

The periodontium consists of the investing and supporting tissues of the tooth (gingiva, periodontal ligament, cementum and alveolar tissue). It has been divided into two parts, the gingiva and the attachment apparatus, which consists of the periodontal ligament, cementum and alveolar bone (Itoiz and Carranza, 1996).

1.2.2 The gingiva

The gingiva's main function is protection of the underlying teeth. The gingiva is tooth dependent i.e. when the tooth is extracted the gingiva disappears. Healthy gingiva is pink, firm, knife-edged and scalloped to conform to the contours of the teeth. The gingiva is divided into two zones, the marginal gingiva and the attached gingiva. The marginal gingiva forms a cuff 1-2 mm wide around the neck of the tooth and is the external wall of the gingival crevice, which is 0-2 mm deep. The surface of the gingival margin is smooth, in contrast to that of the attached gingiva, from which it is demarcated by an indentation called the 'free gingival groove'. The attached gingiva extends from the 'free gingival groove' to the mucogingival junction where it meets the alveolar mucosa (Figure 1.2.1). The attached gingiva is tightly bound to the alveolar bone (Manson and Eley, 2000).

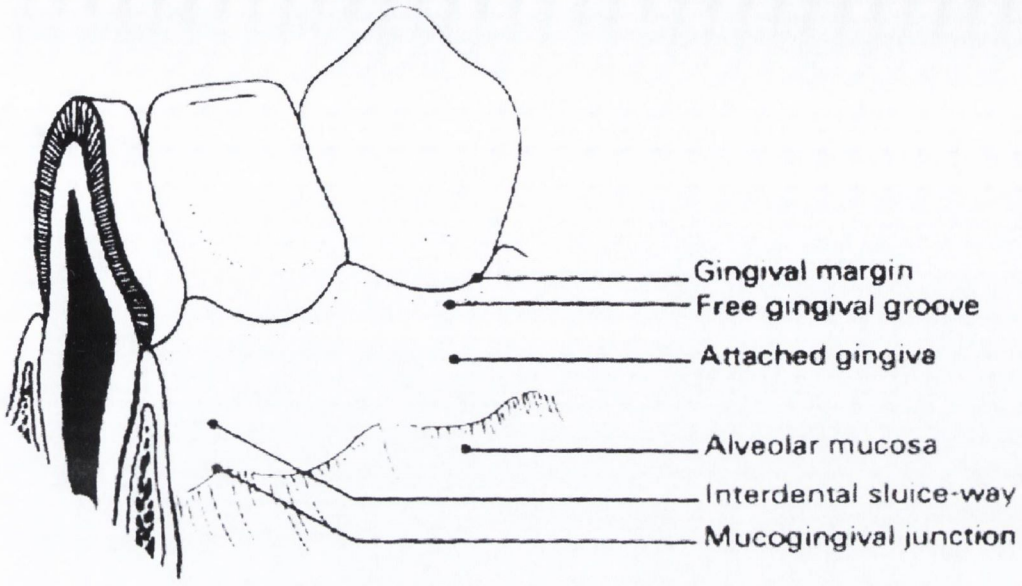


Figure 1.2.1 Illustration of the anatomical features of the gingiva (Manson and Eley, 2000).

1.2.3 The periodontal ligament

The periodontal ligament attaches the root of the tooth to its socket in alveolar bone. The thickness of the ligament varies from 0.1-0.3 mm. The ligament consists of collagen fiber bundles, about 5 μm in diameter, in a ground matrix through which vessels and nerves course (Manson and Eley, 2000). The terminal portions of the fibers that insert into the cementum and bone are known as 'Sharkeys fibers'. There are 6 principal fiber groups in the periodontal ligament:

- Transseptal group
- Alveolar crest group
- Horizontal group
- Oblique group
- Apical group
- Interradicular fibers

The ground matrix consists of two main components, glycosaminoglycans such as hyaluronic acid and proteoglycans such as fibronectin and laminin. The physical functions of the periodontal ligament include:

- Provision of a soft tissue 'casing' to protect the vessels and nerves.
- Transmission of occlusal forces to the bone
- Attachment of teeth to the bone
- Maintenance of the gingival tissues in their proper relationship to the teeth
- Resistance to the impact of occlusal forces

(Carranza and Bernard, 1996)

1.2.4 Cementum

Cementum is the calcified connective tissue that covers the root dentine and the periodontal fiber bundles are inserted into it. It is pale yellow and softer than dentine. Cementum is formed slowly throughout life and is resistant to resorption, it is avascular and without enervation. It consists of collagen fibers embedded in a calcified organic matrix. It contains 65% inorganic material, mainly hydroxyapatite (Manson and Eley, 2000). There are two types of cementum, acellular cementum and cellular cementum. The former covers the cervical third of the root and contains the Sharkeys fibers, which are completely calcified. The latter contains cells (cementocytes) and is less calcified (Carranza and Bernard, 1996).

1.2.5 Alveolar bone

The part of the maxilla and mandible that supports and protects the teeth is known as alveolar bone. It also gives attachment to muscles and acts as a reservoir for ions, in particular calcium.

1.3 Periodontal disease

Gingival and periodontal diseases have been discovered through paleopathology in early humans including the Ancient Egyptians. The Egyptians offered a number of prescriptions for strengthening teeth and gums made with various plants and minerals, and applied to the gums in the form of a paste with honey or vegetable gum as a vehicle (Shklar and Carranza, 1996).

The clinical difference between gingivitis and periodontitis is that while gingivitis shows inflammation there is no attachment loss, whereas there is attachment loss present with periodontitis (Figure 1.3.1). Periodontal disease is a localized inflammatory disease (Southard and Godowski, 1998) due to a local bacterial infection with a pathogenic microflora arising from the accumulation of subgingival plaque within the periodontal pocket (Finkelman and Williams, 1998). Untreated periodontitis results in the loss of the supporting structures of the tooth through resorption of alveolar bone and loss of periodontal ligament attachment. As the disease progresses, the periodontal pocket gets deeper with further destruction of the tooth supporting structures, often resulting in tooth loss (Southard and Godowski, 1998). Table 1.3.1 outlines the different diseases and conditions that can affect a healthy periodontium.

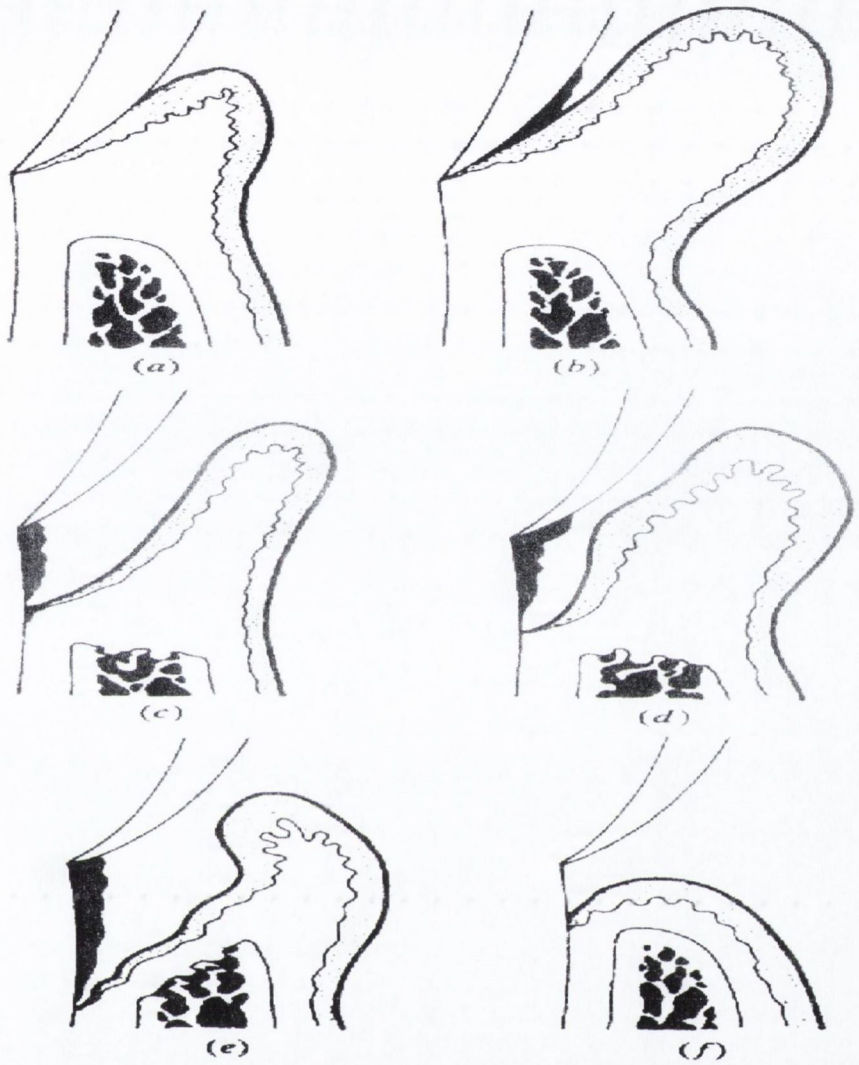


Figure 1.3.1 Various forms of periodontal pathology (a) gingival health and shallow gingival crevice (b) gingival swelling with production of a 'false' or gingival pocket (c) 'true' or periodontal pocket with apical migration of crevicular epithelium and associated resorption of alveolar crest to form a 'suprabony' pocket (d) a suprabony pocket plus gingival hyperplasia producing a deep pocket but little bone loss (e) an 'infrabony' pocket where the epithelial attachment is apical to the alveolar crest (f) gingival recession (Manson and Eley, 2000).

Table 1.3.1 Classification of periodontal disease and conditions.

(adapted from Novak, 2002).

Condition	***Types***	***Notes***
Gingival disease	Plaque induced Non-plaque induced	e.g. pregnancy and diabetes
Chronic periodontitis	Localized (< 30% sites involved) Generalized (>30% sites involved)	Slow to moderate disease progression
Aggressive periodontitis	Localized Generalized	Rapid disease progression
Periodontitis as a manifestation of systemic disease.		Leukaemia, Pappillon-Lefévre syndrome, Down syndrome
Necrotizing periodontal disease	Necrotizing ulcerative gingivitis Necrotizing ulcerative periodontitis	Associated with smoking and immunosuppression.
Abscesses of the periodontium	Gingival abscess Periodontal abscess Pericoronal abscess	

Periodontitis is a widespread condition, in the UK 69% of dentate adults have early signs of the disease in at least one site and only 5% are completely free of any of the clinical signs of chronic adult periodontal disease. In the US over 90% of dentate individuals aged 13 or over have experienced some clinical loss of attachment, although only 15% demonstrate more severe attachment loss (Preshaw *et al.*, 1999). The main symptoms of periodontal disease reported in subjects younger than 40 years of age were gingival bleeding and gingival enlargement, while in those greater than 40 years it was tooth mobility and tooth migration. However the most common reason for seeking treatment is that a dental practitioner has told them that they have gum disease and

require treatment (Brunsvold *et al.*, 1999). Periodontal disease is diagnosed from the assessment of periodontal pocket charting, gingival inflammation and plaque accumulation (Reddy *et al.*, 2000).

1.3.1 Pathogenesis and microbiology

Periodontal diseases are considered infections of the periodontium because there is a bacterial aetiology, an immune response and tissue destruction (Greenstein and Polson, 1998). Listgarten (1986) listed four distinct stages in the inflammatory process (Table 1.3.2). These stages may have periods where they do not progress or may spontaneously reverse.

Stage	*Symptoms*
Initial lesion	Inflammatory lesion with vascular dilation and increased permeability to fluids and blood cells
Early lesion	Lymphocyte rich infiltrate which causes destruction of the local collagen fiber network
Established lesion	Further collagen destruction and epithelial proliferation
Advanced lesion	Evidence of bone destruction, pocket formation and apical migration of the junctional epithelium

Table 1.3.2 Pathology of periodontal disease (Listgarten, 1986).

Bacterial plaque contributes to the pathogenic potential of the subgingival flora due to its ability to colonize and evade host defences, provoking inflammation and tissue damage. However no one single bacteria can be isolated as the causative agent, and it is considered that an increase in any number of these bacteria in the dental plaque can result in the inflammatory reaction seen in periodontitis and gingivitis.

Over 350 species have been isolated from the periodontal pockets of different individuals, but periodontal pathogens considered to be important in periodontitis are included in Table 1.3.3 (Zambon, 1996).

Actinobacillus actinomycetecomitans
Porphyromonas gingivalis
Prevotella intermedia
Treponemes
Eubacterium seburreum
Campyrobacter rectus
Bacteroides forsythus
Peptostreptococcus micros
Selemonas species
Eubacterium species
Streptococcus intermedius

Table 1.3.3 Bacterial species strongly associated with periodontitis.

There are however a number of secondary factors that can contribute also to the accumulation of plaque deposits and prevent their removal. These include:

- Faulty restorations
- Carious cavities
- Food impaction
- Orthodontic appliances
- Misalignment of teeth
- Lack of lip-seal, or mouth breathing
- Developmental grooves on cervical enamel or root surfaces
- Tobacco smoking

1.4 Treatment of periodontitis

1.4.1 Introduction

Historically, the treatment of periodontitis has been largely based on mechanical root debridement (root scaling and planing) to eliminate subgingival plaque. In more recent times the use of antibiotics for periodontitis has begun, based on the experimental evidence that suggests periodontal disease is caused by specific pathogens, mainly anaerobic and gram-negative organisms (Lopéz *et al.*, 2000). There is little or no justification for the use of systemic antibiotics in the treatment of the common forms of adult periodontitis (Palmer, 2001). Antibiotics should be reserved for use in patients who do not respond to conventional mechanical therapy and who continue to exhibit loss of periodontal attachment. Patients with acute or severe periodontal infections may also benefit from antibiotic therapy (American Academy of Periodontology, 1996).

1.4.2 Antibiotics used in the treatment of periodontitis

The main antibiotics used in the treatment of periodontitis are the tetracyclines and metronidazole, in both local and systemic delivery systems. Amoxycillin is also widely used in conjunction with metronidazole systemically. The antiseptic, chlorhexidine, has also been used in a local delivery system.

The tetracyclines used include tetracycline HCl and base, minocycline and doxycycline. Tetracycline is a bacteriostatic agent whose mode of action is inhibition of bacterial protein synthesis. The tetracyclines are broad-spectrum antibiotics, which effect anaerobes and facultative organisms (Greenstein and Polson, 1998). In several investigations the growth of the majority of periodontal pathogens was inhibited by concentrations of tetracycline HCl in the range of a few μg/ml (Table 1.4.1). However these values can be misleading as subgingival plaque bacteria are organized primarily as a biofilm. Antimicrobial action on a biofilm requires concentrations of antibiotic several

times the order of magnitude that is normally required (Tonetti, 1998), due to the synergistic behaviour of the microbes when they coordinate themselves as a biofilm. The biofilm can be several layers deep and while the antibiotic may affect the surface layer, unless concentrations are high enough to penetrate through the biofilm, the lower layers may be left unaffected. Tetracyclines also exhibit anti-inflammatory properties (Müller *et al.*, 1993) through an anti-collagenase effect (Golub *et al.*, 1984).

Table 1.4.1 *In vitro* activity of tetracycline HCl against three commonly isolated periodontopathic bacteria (adapted from Miyake *et al.*, 1995)

	range	***MIC (µg/ml)*** **50%**	***MIC (µg/ml)*** **90%**
P. gingivalis	0.063 – 0.5	0.25	0.5
P. intermedia	0.063 – 0.25	0.125	0.25
A. actinomycetecomitans	0.5 - 8	1	4

Minocycline is a semi-synthetic derivative of tetracycline that has strong binding capacity to root surfaces from where it is released in its active form. It has been shown to have high efficacy against *Actinobacillus actinomycetecomitans* with a MIC several times lower than for tetracycline HCl (Table 1.4.2).

Table 1.4.2 *In vitro* activity of minocycline HCl against three commonly isolated periodontopathic bacteria (adapted from Miyake *et al.*, 1995).

	range	***MIC (μg/ml)* 50%**	***MIC (μg/ml)* 90%**
P. gingivalis	≤ 0.031 – 0.063	≤ 0.031	≤ 0.031
P. intermedia	≤ 0.031	≤ 0.031	≤ 0.031
A. actinomycetecomitans	0.125 –4	0.25	2

Metronidazole in general is effective only against obligate anaerobes, against which it is potent and demonstrates low toxicity. However if there is a mixed infection of anaerobes and facultative organisms it can be ineffective. It has poor activity against *A. actinomycetecomitans* (Table 1.4.3).

Table 1.4.3 *In vitro* activity of metronidazole against three commonly isolated periodontopathic bacteria (adapted from Miyake *et al.*, 1995).

	range	***MIC (μg/ml)* 50%**	***MIC (μg/ml)* 90%**
P. gingivalis	≤ 0.031 - 1	0.5	1
P. intermedia	0.125 - 1	0.25	1
A. actinomycetecomitans	8 - ≥64	64	≥ 64

Chlorhexidine, which is an antiseptic, is also used in the treatment of periodontitis. However chlorhexidine HCl shows much higher MIC (50%) values than any of the antibiotics used. The MIC of chlorhexidine for 52 strains of bacteria ranged from 8 to 500 μg/ml. 64% of the strains tested were inhibited at a concentration of 62 μg/ml. A concentration of 250 μg/ml of chlorhexidine inhibited the growth of all bacteria in 25

subgingival plaque samples investigated, with the MIC ranging from 31 to 250 μg/ml (Stanley *et al.*, 1989).

1.4.3 Systemic use of antibiotics

Numerous studies of the systemic use of antibiotics in the treatment of periodontal disease have been performed and have given some contradictory results. The antibiotics examined include tetracyclines (Müller *et al.*, 1993, Sigusch *et al.*, 2001, Haffajee *et al.*, 1988), metronidazole, penicillins (Winkel *et al.*, 1999, 2001), clindamycin (Sigusch *et al.*, 2001) and fluoroquinolones (Kleinfelder *et al.*, 2000). These have been looked at individually and in combination, metronidazole and amoxycillin (Winkel *et al.*, 2001, López *et al.*, 2000) being the most common combination used. They have been used alone and also as an adjunct to root scaling and planing, or oral surgery.

Haffajee *et al.* (1988) treated patients with destructive periodontal disease using modified Widman flap surgery and systemic tetracycline (1 g/day for 21 days). It was found that in most sites there was a clinical improvement and a decrease in the level of suspected pathogens, with a concomitant increase in the levels of host compatible species. However, none of the suspected pathogens were entirely eliminated from all sites by the therapy employed. The reason for this lack of effectiveness in eliminating pathogens is pointed at in a paper by Sakellari *et al.* (2000). The concentrations of three tetracyclines, doxycycline, minocycline and tetracycline were determined in gingival crevicular fluid (GCF), plasma and saliva. The average concentration at 2 hr was highest in plasma and lowest in saliva. In GCF concentrations were 0.61 μg/ml, 1.49 μg/ml and 1.65 μg/ml for tetracycline, minocycline and doxycycline respectively. However the average GCF concentration in individuals varied widely (between 0 and 8 μg/ml) with approximately 50% of samples not achieving levels of 1 μg/ml. These results suggest that poor absorption of tetracyclines may account for much of the variability in clinical response. Sigusch *et al.* (2001) examined the effect of doxycycline, metronidazole and clindamycin used adjunctively in a 2-step procedure for patients with

rapidly progressive periodontitis (RPP). The first step consisted of root scaling, planing and polishing. The second step included root planing, wound dressing and the beginning of antibiotic therapy. Clinical evaluations examined plaque index (PI), probing depth (PD), clinical attachment level (CAL), and bacteriological sampling. At 6 and 24 months improved PD and CAL were seen in the groups using metronidazole and clindamycin compared to groups using doxycycline and the control. *P. gingivalis* and *A. actinomycetecomitans* were almost completely eradicated in the treatment groups. These results show that metronidazole and clindamycin are effective antibiotics when used adjunctively in a 2-step procedure with root scaling and planing.

Winkel *et al.* (2001) evaluated the adjunctive effects of systemically administered amoxycillin (375 mg TID for 7 days) and metronidazole (250 mg TID for 7 days) in patients who also received supra- and subgingival debridement (test group), *vs.* patients who only received supra- and subgingival debridement (control group). There was a significantly larger change in PD and CAL in the test group than the control group, with both PD and CAL improvements greatest in pockets that had an original PD $\geq$ 7 mm. It was also found that patients diagnosed with *P. gingivalis,* in particular, benefit from this treatment. Winkel *et al.* (1999), also carried out a study examining the effect of amoxycillin in conjunction with clavulanic acid (Augmentin™ dose TID for 10 days) for the treatment of generalized adult periodontitis. Bleeding on probing (BOP), PD and CAL showed no significant change in the test group compared to the control group, and there was no appreciable change in the microbiology of the pockets. Augmentin™ provided no additional benefit in the treatment of adult periodontitis patients. López *et al.* (2000), examined the effect of systemic administration of amoxycillin (500 mg TID for 1 week) and metronidazole (250 mg TID for one week) as the sole therapy in progressive adult periodontitis. No effort was made to change the patient's oral habits and no additional therapy was given. Differences were assessed every 2 months between the test and control group. After 2 months and thereafter, the test group showed significant clinical improvement while the placebo group showed a progressive

deterioration. At 12 months the test group showed a significant increase in CAL, a significant decrease of active sites and a significant reduction in PD.

Kleinfelder *et al.* (2000) examined the use of fluoroquinolones (2 x 200 mg/d for 5 days) in conjunction with open flap surgery (test), against only flap surgery (control) in the treatment of *Actinobacillus actinomycetecomitans (Aa)* associated periodontitis. At 3 and 12 months, the probing depths and clinical attachment level had changed as outlined in Table 1.4.4. *Aa* was suppressed below detectable levels in all test patients, whereas *Aa* could be recovered in 8 of 10 control patients.

Table 1.4.4 PD and CAL values obtained by Kleinfelder *et al.* (2000).

	PD (mm)			*CAL (mm)*		
	0	3 months	12 months	0	3 months	12 months
Test	6.8 (±1.3)	3.6 (±1.0)	3.8 (±1.1)	7.5 (±1.4)	5.4 (±1.4)	5.5 (±1.3)
Control	6.5 (±0.7)	4.0 (±1.7)	4.1 (±1.6)	7.5 (±1.0)	6.3 (±1.7)	6.4 (±1.8)

The overall benefit of systemic antibiotics can be difficult to ascertain due to the number of variables in each of the studies. These include:

- The type of periodontitis being treated
- The adjunctive treatment used
- The antibiotic used
- The dosage of the antibiotic
- Compliance

However it would appear that certain combinations of antimicrobial agents do offer a clinical benefit in the treatment of certain types of periodontitis.

1.4.4 Local delivery systems

The following five concepts are important for a locally delivered drug to be effective

- It has to kill or inhibit the target microorganism
- It must reach the desired site of action in appropriate concentrations
- It must remain at the site for a sufficient period of time
- It must cause no harm to the surrounding environment

There are three basic approaches to localized oral therapy: mouthrinses, subgingival irrigation, and periodontal application of controlled delivery systems.

- Mouthrinses - According to Eakle *et al.* (1986) these do not reach the subgingival area and are therefore ineffective. Pitcher *et al.* (1980) reported that there was lack of penetration of the gingival crevice by mouthrinsing, and agents in mouthrinses do not predictably reach beyond 5 mm into the periodontal pocket irrespective of pocket depth. Although rinsing alone has been found to be an ineffective means of penetrating into periodontal pockets, mouthrinses may be suitable for the treatment of supragingival plaque and gingivitis (Flemmig *et al.*, 1990).

- Subgingival irrigation - Eakle *et al.* (1986) demonstrated that supragingival irrigation using an oral irrigator could deliver a disclosing solution 44% to 71% of pocket depth. According to Pitcher *et al.* (1980) irrigating solutions delivered directly intracrevicularly can predictably reach deep periodontal pockets; however, the efficiency of penetration by direct irrigation also decreases with increasing pocket depths from 4 to 6 mm. Subgingival irrigation can be divided into two models of delivery. While a patient-applied irrigant can reach pockets 6 to 9 mm deep based on the system used, the professional-applied irrigants can prove more effective (Braun and Ciancio 1992).

- Controlled delivery systems – these should have a duration of drug release that exceeds 1 day. They should be designed to constantly replace the drug cleared by the gingival fluid. Two components of the system are very important in order to achieve this objective: the drug reservoir and the rate controller to regulate drug replacement and maintain constant concentrations of the free drug in the periodontal pocket. In order to establish a drug reservoir of sufficient size in the periodontal pockets, the delivery system should be able to expand the volume of the periodontal pocket and remain dimensionally stable (Tonetti *et al.*, 1994).

Candidate formulations for local controlled release delivery systems should exhibit the following properties (Jones *et al.*, 2000):

- Controlled drug release over a prolonged period of time
- Acceptable mechanical properties to ensure ease of administration
- Mucoadhesive properties to ensure retention of the formulation within the pocket

The concept of local drug delivery to the periodontal pocket began in the late 1970's with a researcher named Goodson who developed a hollow fiber loaded with tetracycline HCl. These small permeable hollow fibers were made of cellulose acetate and were filled with tetracycline hydrochloride (Lindhe *et al.*, 1979). However in a further study examining the *in vitro* release of tetracycline hydrochloride from six different fiber types, it was found that cellulose acetate along with polypropylene, polyethylene, polycaprolactone and polyurethane all released their drug load within 1 day. Ethyl vinylacetate fibers showed release up to 9 days, which was considered a more suitable time period for the treatment of periodontitis (Goodson *et al.*, 1983). In a further paper of Goodson *et al.* (1985), monolithic fibers made of ethylene vinylacetate and loaded with 25% tetracycline hydrochloride provided sustained release for 10 days under *in vitro* test conditions. The fiber consisted of a polymeric matrix loaded with

tetracycline HCl powder. The mixture was then extruded at high temperature as a melt, to produce a cylindrical fiber. This ethyl vinylacetate fiber was eventually launched commercially under the trade name *Actisite*™. *Actisite*™ is applied by inserting the fiber into the periodontal pocket in successive layers until the pocket is completely filled. A last wrap is then put around the tooth to help maintain the fiber in-situ. The fibers are not resorbable and must be removed after 10 days. The application of this product is time consuming, taking up to 15 min to apply the fiber to one tooth (Tonetti *et al.*, 1994).

A second product that has been developed and launched on the market is *Periochip*™. This chip is an orange-brown rectangular wafer rounded at one end. It measures 4 mm by 5 mm by 0.35 mm. It weighs 7.4 mg and contains 2.5 mg of chlorhexidine gluconate in a biodegradable matrix (Killoy, 1998). Release of the chlorhexidine from the matrix is altered by the amount of chlorhexidine incorporated into the film, by the cross-link density of the polymer (gelatin), and by the chlorhexidine salt used. This chip is biodegradable which is an advantage over *Actisite*™, however anecdotal evidence suggests its premature loss from the periodontal pocket is a frequent problem.

Another commercially available product is metronidazole dental gel, which contains 25% w/w metronidazole in a glyceryl monoleate and sesame oil gel (*Elyzmol*™). This product is formulated as a suspension, however on contact with gingival fluid it becomes a controlled release semi-solid. This system is based on the ability of mixtures of monoglycerides and triglycerides to form liquid crystals when in contact with water. Metronidazole is used in the form of metronidazole benzoate, which further helps slow release. Dissolution studies were performed on a number of systems containing different concentrations of metronidazole benzoate. The longest release obtained was 48 hr, with most of the systems showing 100% release at 24 hr (Norling *et al.*, 1992). The product has many positive properties including the fact that it is syringeable and biodegradable, however the release of drug is rapid.

A 2-component biodegradable controlled release delivery system containing doxycycline hyclate has been formulated *(Atridox™)*. The components of the system are contained in two separate syringes that are mixed just prior to use. One syringe contains doxycycline hyclate and the other the polymer of polylactide acid, poly (D-L-lactide) dissolved in a biocompatible solvent, N-methyl-2-pyrrolidone (NMP). When the doxycycline hyclate is injected into the periodontal pocket the NMP carrier, which is highly miscible in a non-aqueous environment is replaced progressively by water, which causes the polymer to return to its solid state. The retention of the product is enhanced with one of two periodontal dressings (non-eugenol or 2-octyl cyanoacrylate). As the polymer degrades the doxycycline is gradually released. The dressing used seemed to strongly affect the results achieved with a mean concentration at day 7 of 309 μg/ml for the non-eugenol group and 148 μg/ml for the octyl CA group. The concentration of the drug in gingival crevicular fluid (GCF) fell rapidly after day 7. In the non-eugenol group it was 309.35 μg/ml at day 7 and 15.72 μg/ml at day 8, while in the octyl CA group it was 147.60 μg/ml at day 7 and 36.32 μg/ml at day 8 (Stoller *et al.*, 1998).

Two products using poly (ortho esters) have been developed. The first was prepared by the condensation of 1, 2, 6-hexanetriol and an alkyl orthoacetate, giving a semisolid material. When tetracycline was incorporated, complete release occurred within 24 hr, but when small amounts of $Mg(OH)_2$ were incorporated drug release could be extended to many weeks. A 0.5% drug loading gave a 10-day release (Roskos *et al.*, 1995). The second type developed consisted of poly (ortho esters) (POE) containing lactic acid (LA) with the formula POExLAy, where x = 95 and y = 5, the product was a solid and where x = 70 and y = 30 the product was semi-solid. Both formulations were loaded with tetracycline base. It was found that the greater the LA content the faster was the release. $POE_{95}LA_5$ showed no release for the first 48 hr, thereafter it gave almost zero order release up to 100% drug release at 2 weeks, (Schwach-Abdellaoui *et al.*, 1999). This product shows promising release profiles but due to the content ratio it is a solid so application and retention in the pocket could prove difficult. $POE_{70}LA_{30}$, which is a semi-solid, released 100% of its drug within 10 days and had a shorter lag time.

However when examined *in vivo*, its retention was poor, with only 7/20 sites positive for tetracycline at day 4, 5/20 at day 7 and 2/20 at day 11 (Schwach-Abdellaoui *et al.*, 2001).

Collagen preparations have also been examined for use in localized periodontal therapy. Cross-linked and non cross-linked collagen films containing tetracycline and a collagen sponge containing tetracycline were formulated. It was seen that the cross-linked films released adequate levels of tetracycline for up to 10 days (Minabe *et al.*, 1989). Another product developed consisted of a soluble insert with a fast-release and a sustained-release part containing ofloxacin as the antibacterial agent. The insert was a thin strip 1 mm in width and 0.4 mm in thickness, and contained a ratio of 54:46 of sustained release particles to fast release particles. The carrier material for the sustained release particles was methacrylic acid, while for the fast release it was hydroxypropylcellulose. The *in vitro* release profile showed a bi-phasic pattern, within 1 hr ofloxacin levels reached a peak of 12 mg/ml, gradually decreasing until the third day, thereafter a constant level above 2 µg/ml was maintained from the 3rd to the 7th day (Higashi *et al.*, 1990).

Novel mucoadhesive formulations containing hydroxyethyl cellulose (HEC), *Carbopol*™ and polycarbophil with the drug, metronidazole has been designed for the treatment of periodontal disease (Jones *et al.*, 1997). Properties such as adhesion, drug release, and syringeability were examined. Formulations containing HEC showed the most promising characteristics as a candidate for localized drug delivery to the periodontal pocket. A preliminary clinical evaluation was performed on a formulation containing 3% HEC, 3% PVP and 1% PC, with 5% tetracycline HCl. The clinical efficacy was compared to an identical formulation minus the tetracycline. One week following administration there was a significant reduction in numbers of subgingival microbial pathogens (Jones *et al.*, 2000).

1.5 Polyoxyethylene-polyoxypropylene co-polymers

1.5.1 Introduction

Polyoxyethylene-polyoxypropylene co-polymers *(poloxamers)* were commercially introduced in the early 1950's. They are synthesized in a two-step process:

- Creation of a hydrophobic core with the desired molecular weight by the controlled addition of propylene oxide to propylene glycol.
- Subsequent addition of ethylene oxide units that are inserted on both sides of the hydrophobic nucleus. The process is carried out with basic catalysis and under conditions of high temperature and pressure.

Depending on the synthetic process, total molecular weights can range from 1,000 - 16,000 Da and the hydrophilic segment can comprise 15-90% of the copolymer. *Poloxamers* are defined by number. The first two digits of the number when multiplied by 100 correspond to the approximate average molecular weight of the polyoxypropylene portion of the molecule. The third digit when multiplied by 10 corresponds to the percentage by weight of the polyoxyethylene portion. Water solubility is variable and depends on their hydrophobicity and molecular weight. Solubility is greater in cold water and is due to the formation of hydrogen bonds between the water and the oxygen atoms of the molecule (Garcia-Sagrado *et al.*, 1994).

Poloxamer 407 has been applied pharmaceutically to a number of uses in the last number of years. Veyries *et al.* (1999) investigated using *poloxamer 407* 25% w/w to prolong the residence time of vancomycin, a time dependent antibiotic, in a body site with a high infectious risk. In rats a single does gave a high local concentration for 24 hr, followed by lower but effective antibacterial levels for at least 8 days. Scherlund *et al.* (2000) investigated thermosetting microemulsions and micellar solutions as drug delivery systems for anesthetizing the periodontal pocket and determined that they have several advantages over emulsion based formulations including good stability, ease of

preparation and improved handling. Esposito *et al.* (1996) developed a tetracycline containing product with *poloxamer 407* for direct periodontal pocket administration, for the treatment of periodontitis. *In vitro* release studies showed 65% release after 7 hr. This was not a good sustained release so while the system was a good formulation for delivery it did not offer an adequate release profile.

1.5.2 Thermoresponsivity

The structure of the *poloxamer* with a hydrophobic core and hydrophilic chains attached confers surface active properties on the copolymer. This surface activity promotes micellar association between the molecules. *Poloxamers* above a certain molecular weight are able to form gels in an aqueous solution, at varying concentrations. *Poloxamer* 184 to 188 form gels from concentrations of 50 to 60%, *poloxamer* 407, however can form gels from concentrations as low as 20%. The main characteristic of these gels is their thermoreversible property. The reason for this thermoresponsivity is not fully understood and a number of theories have been put forward to explain it. One such theory is that as the temperature increases, micellar entanglement is promoted, leading to gel formation and an overall increase in bulk viscosity.

1.5.3 Effect of added excipients

The gelation properties of *poloxamer* aqueous solutions can be varied by the addition of different substances e.g. sorbitol, sucrose and polypropylene glycol decrease gelation temperature. Polyethylene glycols (PEG) and sodium dodecyl sulphate increase gelation temperature. The increase in gelation temperature seen with PEG may be due to the fact that the presence of the PEG causes some modification of the process of micellar association and also the PEG molecules may form mixed micelles with the copolymer. Thus in the presence of PEG, *poloxamer* micellar association is hindered and a mixed micellar system with different physiochemical properties is formed, leading to an increase in gelation temperature. A decrease in gelation temperature is seen with the

homologous series of para-hydroxybenzoate esters, methyl, ethyl, propyl and butyl, with the more lipophilic ester producing a greater decrease in the gelation temperature. This can be explained by the hydrophobic binding ability of the esters for the *poloxamer* chains, with the more lipophilic ester having a greater binding ability. It is thought that the esters bind to the polyoxyethylene chains, promoting dehydration and causing an increase in entanglement of adjacent micelles, producing gelation at lower temperatures (Gilbert *et al.*, 1987).

1.5.4 Poly-alkyl-2-cyanoacrylates

The alkyl-2-cyanoacrylates are biodegradable tissue adhesives. 2-cyanoacrylates are acrylic monomers that have the chemical structure shown in Figure 1.5.1.

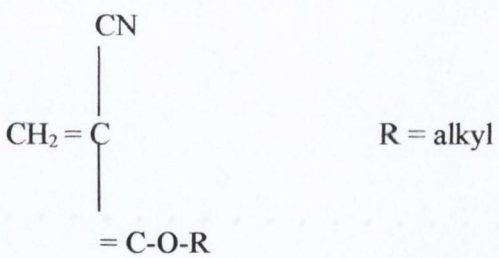


Figure 1.5.1 Chemical structure of 2-cyanoacrylates.

They are synthesized from formaldehyde and alkyl cyanoacetates. The monomers are highly polymerizable under neutral and basic conditions. To prevent polymerization occurring during synthesis and storage they are distilled under acidic conditions, using SO_2. The detailed mechanism of their polymerization is not yet fully understood, but it seems probable that they polymerize via an ionic mechanism in which water molecules act as co-initiator.

Their rate of degradation is dependent on the size of their alkyl group. The rate of degradation is the greatest for methyl-α-cyanoacrylate and diminishes as the size of the alkyl group increases. Cyanoacrylates with branched alkyl groups degrade faster than

their straight chain homologs, with degradation being faster in alkaline solutions. In the presence of an aqueous environment, the polymer gives rise to the degradation product formaldehyde, which is histotoxic and can cause acute and chronic inflammation unless accumulated levels are below determinable thresholds. The cytotoxicity of cyanoacrylate polymers is highly dependent on the biodegradation rate of cyanoacrylate polymers and hence on the formaldehyde production rate.

1.6 Halloysite

1.6.1 Morphology

Halloysite was named by Berthier in 1826 and was at that time regarded as an amorphous substance. However later x-ray studies showed that it had a crystalline nature. Electron micrographs obtained by Shaw and Humbert (1941) showed that the mineral typically occurred as split rods. However it was work by Bates *et al.* (1950) which deduced its currently accepted tubular structure.

Halloysite is a 1:1 layered aluminosilicate clay. Halloysite morphology mostly consists of ultramicroscopic multi-layered hollow cylinders, the walls of which are formed from alternating layers of alumina and silica (Figure 1.6.1). The alternating alumina and silica layers demonstrate a packing disorder causing them to curve. These deformed layers make up the wall of a roughly cylindrical shape. Samples of halloysite consist of a mixture of lengths and diameters as well as nontubular clay material. Commercial samples of halloysite showed microcylinders ranging from 40 nm to 0.3 μm in diameter and from 1-20 microns in length. The inner cores ranged from 16-50 nm in diameter, however many of the cylinders do not appear to have a single unique lumen, but rather a series of voids along the axis between the layers composing the wall (Price and Gaber, 1997). Morphology of the clay can vary greatly from deposit to deposit, and within deposits, so applications where strict conformability is required may mean that the halloysite must be taken from only one source.

1.6.2 Sourcing halloysite

New Zealand China Clays Ltd. (subsidiary of Ceramaco Corporation Ltd.) is the principal commercial source of halloysite. The deposits were initially discovered in the early 20th century, with the largest resources in the North Island (Maturi Bay, Mahimahi, Maungaparerua) and several smaller resources in the Coromandel region (Harvey, 1996). Other commercial sources of halloysite include Turkey, Japan and South Korea. The Czech republic is another minor source while in France there is a deposit located in Dordogne. In the USA deposits are found in Indiana and Nevada.

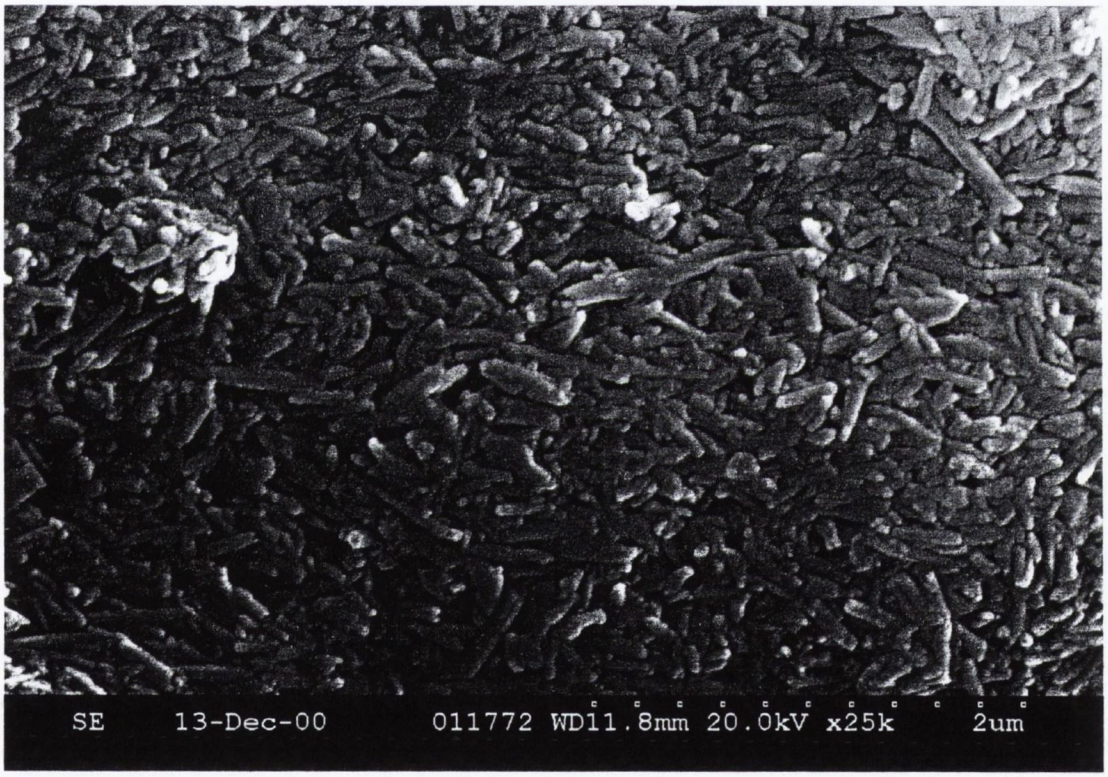


Figure 1.6.1 Scanning electron microscopy (SEM) of halloysite.

1.6.3 Origin of halloysite

Halloysite is a product of soil weathering, which arises due to the weathering of rhyolitic tephras, where volcanic glass alters to allophane, which on ageing crystallizes to cylindrical halloysite. This weathering sequence also occurs in Japanese volcanic soils but at a faster rate than New Zealand deposits (Kirkman, 1975). Kirkman (1977) examined the crystal growth patterns of the halloysite mineral, which implied that the cylindrical halloysite particles begin to crystallize from allophane after 11,000 years, and that the initially very small and poorly formed particles grow with age and improve in crystallinity. The extent to which halloysite is formed by the weathering process described above, is dependent on a number of factors, including silica concentrations in soil and the soil temperature (Farmer *et al.*, 1979)

1.6.4 Applications of halloysite

Price *et al.* (2001) examined the use of halloysite as a low cost alternative to microencapsulation. A hydrophilic agent, oxytetracycline HCl, a hydrophobic agent, khellin and the co-enzyme, nicotinamide adenine dinucleotide (NAD), were drug loaded into halloysite and their release profiles determined. Preliminary experiments for hydrophilic compounds showed the release rate to be rapid, with an initial burst of 20% oxytetracycline HCl released, followed by fairly rapid release over 30 hr. In order to slow release, the halloysite was blended with a polymeric carrier, epoxy Quitol 651. This reduced the burst to 5% and gave a monophasic release for 90 hr. Khellin showed a burst release of 2% with a very slow release thereafter, only 7% of the drug being released over a 192 hr period. This is due to both the drug's inherent insolubility in water and the fact that it remains as a hydrophobic solid under the experimental conditions. NAD was used to examine the difference between two different grades of halloysite, a premium and 'G' sample. The 'G' sample contained substantially more small cylinders than the premium sample, which contained 59% non-tubular material. Loading efficacies were 7.38% in the premium grade *vs.* 19% in the 'G' grade, pointing

to the importance that the grade of halloysite can have on the loading efficacy and subsequent active release.

Lvov *et al.* (2002) used halloysite in conjunction with poly(ethyleneimine) (PEI) to assemble multilayers of the halloysite glued together with PEI. The multilayers were from 2 up to 20 layers. Halloysite loaded with NAD was used to assemble a four-unit multilayer (PEI/halloysite/PEI/ADH). The ADH (alcohol dehydrogenase) catalyzes the oxidation of alcohols to aldehydes in the presence of the co-factor NAD.

1.7 Saliva

1.7.1 Saliva production

Saliva is produced by three pairs of salivary glands, the parotid, submandibular and sublingual glands, which account for 90% of saliva produced. The parotids are the largest salivary glands and are situated in front of the ear, with a duct opening into the oral cavity in a papilla opposite the second upper molar tooth. The submandibular gland is about half the size of the parotid gland and is found between the body of the mandible and the mylohoid muscle (which forms the floor of the mouth). It opens into the floor of the mouth. The sublingual is the smallest of the major salivary glands, being one-fifth approx. the size of the submandibular. Smaller glands embedded in the mucosa and submucosa of the mouth, pharynx and cheeks account for the other 10% of saliva produced. References quote varying daily saliva volumes from 0.5 L (Whelton, 1996) to 1.5 L (Rowe *et al.*, 1995). Under resting conditions secretion is in the region of 0.3-0.5 ml/min.

Salivation is controlled mainly by the salivary center located in the medulla and pons, through the automatic nervous system. Each gland is composed of a well vascularised system of acini and ducts, with the glands being further divided into lobes and lobules by connective tissue. The major glands involved in salivary secretion are innervated by

parasympathetic and sympathetic nerves, with the type and quantity of saliva differing depending on the type of stimuli. Parasympathetic stimulation results in a rapid and abundant secretion of low viscosity saliva, rich in enzymes, whereas sympathetic stimulation gives thicker more mucinous saliva produced in smaller quantities. A number of factors can affect the unstimulated flow rate of saliva and these are outlined in Table 1.7.1.

Table 1.7.1 Factors affecting salivary flow rate (adapted from Davies, 1996).

	Factors affecting unstimulated salivary flow rate	***Effect of factor on salivary flow rate (SFR)***
1.	Degree of hydration	Body water content decreased by 8%, salivary flow rate is reduced to ~0%
2.	Body position	Standing increases salivary flow rate while lying down reduces it relative to sitting
3.	Exposure to light	Salivary flow rate decreases by 40% in the dark
4.	Circadian rhythms	Salivary flow rate decreases during sleep
5.	Sight, smell or thought of food	Increase in salivary flow rate
6.	Fear	Reduced salivary flow rate
7.	Drugs	Can increase e.g. clozapine or decrease salivary flow rate e.g. hyoscine

1.7.2 Salivary composition

Saliva is composed of 99.5% water and 0.5% protein and electrolytes together with various bactericidal and antimicrobial components. It consists of two major secretions, firstly a serous fluid containing bactericidal substances (Table 1.7.2). Antimicrobial substances present in saliva are an important part of the mouth's defense mechanism against invasion by pathogenic bacteria.

The second secretion is mucus, which is composed of mucin and water. The constant production of saliva also helps wash away bacteria and food debris. Its antibody and antimicrobial content help inhibit dental caries and oral infection. Its lubricant qualities prevent epithelial dehydration, facilitate chewing and swallowing, contribute to taste perception and aid speech. The calcium phosphate and hydroxyl ions present in saliva help prevent dental caries, while bicarbonate acts as a buffer.

Table 1.7.2 Anti-microbial factors found in saliva.

	Anti-microbial (non-immunoglobulin) factors found in saliva
1.	Salivary peroxidase
2.	Myeloperoxidase
3.	Lysozyme
4.	Lactoferrin
5.	Aggregating factors
6.	Amylase
7.	Anionic proteins

1.7.3 Salivary mucin

Salivary mucins are synthesized by the mucous acinar cells of the submandibular and sublingual glands. They are high molecular weight glycoproteins composed of a protein backbone rich in hydroxyamino acids (threonine and/or serine) and proline. Numerous carbohydrate side-chains are linked O-glycosidically to many of the hydroxyamino acids. In terms of structure there are two distinct forms of salivary mucins, those that are composed of multiple covalently bonded subunits and are as a result very large and secondly those that consist of single polypeptide chains and are therefore small. Both mucin forms are found in human saliva and are referred to as MG1 and MG2

respectively. It is though that MG1 is more involved in a coating function, whereas MG2 interacts with microorganisms (Tabak, 1995).

1.8 Xerostomia

1.8.1 Signs and symptoms

Xerostomia is a condition characterized by a reduction or loss in salivary flow often with a concurrent change in the composition of the saliva, resulting in dryness of the oral cavity. This in turn leads to changes in oral pH and oral microflora leading to increased risks of infection, dental caries, mucositis, gingivitis and periodontitis (Plankhurst *et al.*, 1996). There is also an increased risk of *Candida* infections and angular cheilitis (Chaushu *et al.*, 2000). Xerostomia may also lead to difficulties with some or all of the following, polydypsia, polyuria, sleeping, speaking, swallowing and mastication (Hamlet *et al.*, 1997). There may also be changes in taste acuity (Mossman and Henkin, 1978).

There are a number of conditions that can result in the development of xerostomia. The most common are radiation treatment of the head and neck region, Sjögrens syndrome and drug treatment. However other conditions have been reported to cause xerostomia including HIV (Schiødt *et al.*, 1992), brain tumours and neurosurgical procedures (Ettinger, 1996). Xerostomia while not considered a serious illness, can result in symptoms which patients find very distressing. The condition is often associated with serious or terminal illnesses and therefore causes further distress to these patients.

1.8.2 Sjögrens syndrome

Sjögrens syndrome is named after Henrik Sjögren (1899-1986), an ophthalmologist in Sweden, who in 1933 described the clinical and pathological features of the disease in 19 women, most of whom were post menopausal and 13 of whom had arthritis (Parke and Buchanan, 1998). Sjögrens syndrome is an autoimmune condition that can either occur

independently (primary Sjögrens) or in association with other autoimmune conditions or other connective tissue diseases (secondary Sjögrens). The European classification defines it as a chronic, autoimmune, inflammatory disease of exocrine glands, characterized by diminished salivary and lacrimal gland function. It may occur at any age, but it primarily affects females (9:1) in there 50's and 60's. Patients with Sjögrens disease, especially primary Sjögrens have a 40-times greater risk of developing malignant lymphoma, and there have also been reported associations with neoplastic and myeloplastic syndromes, fibromyalgia and chronic fatigue syndrome (Parke and Buchanan, 1998).

Symptoms of Sjögrens syndrome can range from dryness of mouth (xerostomia), eyes (keratoconjunctivitis sicca), skin and vaginal mucosa to serious systemic problems such as vasculitis along with renal involvement. Recently there has been also a relationship observed between Sjögrens syndrome and neurological conditions e.g. peripheral neuropathy (Lafitte, 2000). The problems associated with dry eye are frequently mild, with the most common complication being secondary bacterial infection due to a deficiency in lysosome. If this goes untreated it may result in perforation of the cornea or limbal ulceration.

Xerostomia associated with Sjögrens syndrome results in all of the problems listed in section 1.8.1, with dysphagia, fissuring of the lips, Candidiasis, oral ulcers and dental caries being observed (Parke and Buchanan, 1998, Soto-Rojas *et al.*, 1998). However the xerostomia is considered to be more severe than would be seen with drug-induced xerostomia and patients perceive the oral dryness as intense and distressing (Soto-Rojas *et al.*, 1998). Also with Sjögrens syndrome parotid gland enlargement will be observed and is usually bilateral. The enlarged parotid glands can become infected, usually with *Staphylococcus aureus*. Histologically, the salivary glands of Sjögrens syndrome show acinar atrophy and lymphoid infiltration (Parke and Buchanan, 1998). On clinical examination of the oral cavity, it will be dry and glazed, with fine wrinkles. The tongue typically has a characteristic lobulated red surface, with partial or complete

depapillation. Soreness and redness is usually due to Candidiasis, and often there will be evidence of severe dental caries and periodontal disease. Rheumatoid arthritis is the most common associated connective tissue disease, but other connective tissue diseases associated with Sjögrens are systemic lupus dermatomyositis and primary bilial cirrhosis (Scully, 1986).

1.8.3 Radiation therapy

Radiation therapy is a common treatment for head and neck cancer. Often with radiation of the head and neck, the salivary glands incidentally lie in the line of irradiation. Xerostomia can occur as a result of damage to the salivary glands, and is dose related. With average doses of 60-70 Gy being used for the treatment of oral carcinoma and doses as low as 20 Gy causing cessation of salivary flow (Scully and Epstein, 1996), with little improvement on cessation of treatment, this is a significant cause of distress among patients. In a study where full dose irradiation (65-74 Gy) was used, 79% of parotid glands had no output, while with doses of 40-52 Gy 72% of parotid glands still produced some saliva (Funegård *et al.*, 1994). Indeed the stimulated whole saliva flow rate can be reduced by up to 83.3% over a 6-week treatment period (Dreizen *et al.*, 1976). In many cases the damage to the parotid glands is essentially irreversible with no output up to 2 years post radiation therapy (Henson *et al.*, 1999). However there can be some recovery of salivary function where not all of the salivary glands lay in the line of radiation and the dose of irradiation involved was low. Partially irradiated glands obviously have increased chance of recovery as some of the acinar cells are left functional (Joyston-Bechal, 1992). Patients with advanced Hodgkin's disease or non-Hodgkin's lymphoma in particular tend to develop more severe xerostomia due to combination chemoradiotherapy consisting of total neck irradiation and chemotherapy (Kosuda *et al.*, 1999).

Histologically the damage caused to the salivary glands includes marked acinar atrophy and chronic inflammation, with the serous acini considered to be the most radiosensitive

followed by the mucous acini (Garg and Malo, 1997). This results in the production of viscous saliva at the start of treatment followed by decreasing production. There are also changes in salivary components and consistency with a decrease in buffering capacity and pH, and an increase in levels of sodium chloride (Dreizen *et al.*, 1976). The saliva becomes increasingly viscous, losing its lubricating properties, causing membrane dehydration, pain and tenderness due to damage to the serous acinar cells. There is also increased risk, as with Sjögrens syndrome of developing conditions such as Candiadisis and dental caries, with cariogenic microorganisms becoming more prevalent as saliva production is reduced (Chaushu *et al.*, 2000, Brown *et al.*, 1975). There can also be problems with swallowing and mastication (Hamlet *et al.*, 1997) and with taste acuity (Mossman and Henkin, 1978).

1.8.4 Drug therapy

There are over 400 drugs which state xerostomia as a side effect (Ettinger 1996). Saliva secretion is stimulated by the parasympathetic autonomic nervous system. Drugs with an anticholinergic or sympathomimetic activity may cause a reduction in saliva output (Korstanje, 1995). The most widely used of these drugs are listed in Table 1.8.1. The intensity of the xerostomia will vary from patient to patient, and will also depend on a number of factors such as, dose of drug and duration of administration (Ettinger, 1996). Chemotherapy while having a short-term effect on salivary function appears to cause no long-term damage, however when used in combination with radiation therapy it can cause increased damage to radiation-induced injury (Kosuda *et al.*, 1999).

1.8.5 Other causes of xerostomia

While Sjögrens syndrome, radiation therapy and drug therapy are the most common causes of xerostomia, there are a number of other conditions that can result in xerostomia. Diabetes mellitus, which has been poorly controlled over a long period of time, has been linked to causing a reduction in salivary flow (Chavez *et al.*, 2000).

More recently the development of a HIV-associated salivary gland disease, characterized by enlarged major salivary glands and/or xerostomia has been observed. This HIV-associated salivary gland disease can clinically resemble Sjögrens syndrome (Schiodt *et al.*, 1992). There is also a percentage of the population, especially among older groupings who suffer from xerostomia, but have no pathological reason for the condition.

Table 1.8.1 Commonly used drugs that can cause xerostomia.

Anti-pyschotics	***Antihistamines***	***Tricyclic antidepressants***	***Antiemetics***	***Anticholinergics***
Phenothiazines	Promethazine	Amitryptylline	Antihistamines	Benzhexol
Chlorpromazine	Diphenydramine	Imipramine	Phenothiazines	Benztropine
Trifluorperazine			Butyrphenones	
Butyrphenone				
Haloperidol				

1.8.6 Diagnosis

The diagnosis of xerostomia can be difficult due to the subjective nature of the condition. The use of a standard questionnaire is widespread. It is also standard to measure the resting and stimulated salivary levels using one of a number of methods (Weisenfeld *et al.*, 1983). Whole resting saliva can be measured by expectoration into a holding device over a defined time period, while for stimulated saliva a substance e.g. paraffin wax or chewing gum is masticated for a period of time and saliva produced is collected.

1.9 Treatment of xerostomia

1.9.1 Factors to be considered

The treatment of xerostomia is dependent on a number of factors, the severity of the xerostomia i.e. does the person have any residual salivary function remaining that can be stimulated, is the xerostomia temporary or permanent? The side effect profile of the chosen treatment must also be considered, while the cost *vs.* effectiveness of the chosen method needs to be evaluated. Treatments currently available vary considerably in effectiveness, cost, ease of use and side effect profile, and in many cases the results are highly subjective.

1.9.2 Salivary stimulants

Pilocarpine is the drug of choice for use as a salivary stimulant. It is only of use in patients where there is still residual function left, with the commercial pilocarpine product *Sialagen™* only registered for use in post irradiation xerostomia (British National Formulary). Where there has been total irradiation of the salivary glands pilocarpine is not an option as there will be no acinar cells left to stimulate, so this treatment has its limitations. The drug also has a poor side effect profile, including sweating, increased urinary frequency, flushing, dizziness and tachycardia, with all side effects being dose related (British National Formulary). Clinically significant benefits can be seen with continuous treatment for 8 weeks using doses greater than 2.5 mg, but side effects were seen to increase with increasing doses (Rieke *et al.*, 1995, Vivino *et al.*, 1999).

1.9.3 Radioprotective agent

A more recent advance in the treatment of xerostomia resulting from irradiation relates to the prevention of xerostomia, as opposed to treatment of the condition and involves

the use of amifostine (Schering-Plough) as a radioprotective agent. Amifostine is licensed in the UK (specialist use only) for the reduction of risk of infection related to neutropenia in patients with ovarian cancer undergoing treatment with cisplatin and cyclophosphamide (British National Formulary). In a study where amifostine was used at a dose of 500 mg/M^2 in 17 patients being treated with high-dose radioiodine therapy there was no incidence of xerostomia, resulting in increased quality of life for patients (Bohuslavizki *et al.*, 1998). However it has a severe side effect profile and can cause hypotension, nausea and vomiting. It is also an extremely expensive drug, costing stg £160 for a 500 mg vial, making it pharmacoeconomically unviable for long term use.

1.9.4 Salivary gland transfer

The other approach to treatment of xerostomia caused by irradiation is salivary gland transfer, where the salivary gland as part of a surgical procedure is moved to an area of the cavity that is outside the proposed radiation field thus preventing xerostomia developing (Naresh *et al.*, 2000). Again this has a number of limitations. It is only an option where surgery is already required as it would not be cost effective as a procedure on its own. It is also a newly developed procedure and it may be a number of years before it is commonly available. Both of the methods described in sections 1.9.3 and 1.9.4 are only of use in patients being exposed to irradiation.

1.9.5 Saliva substitutes

The other option available is the use of artificial salivas or saliva substitutes (oral lubricants). Saliva substitutes are of use in those patients who have little or no residual salivary gland function, or where pilocarpine cannot be used due to side effects or contraindications to its use. There are many saliva substitutes available on the market e.g. *Oralbalance*™, *Mouthkote*™, *Glandosane*™, *Luborant*™ but none are ideal. This is due to the complex nature of saliva making it difficult to produce a product that adequately mimics the properties of saliva. Common criticisms of currently available

oral lubricants include little relief of symptoms, strange consistency, bad taste and short duration of action (Furomoto and Carter-Hanson, 1998). However in their favour they are a cheap, safe and an easy to use option with no problematic side effects. The ideal saliva substitute should be bioadhesive, biocompatible and should also help protect against some of the many problems associated with xerostomia e.g. dental caries, *Candida* infections. It should also help relieve the symptoms associated with the xerostomia e.g. difficulty in swallowing and chewing, talking, etc.

Current saliva substitutes contain either bioadhesive agents e.g. sodium carboxymethylcellulose (NaCMC), xanthan gum or animal mucin e.g. bovine submaxillary mucin (*Saliva Orthana*™) (Levine *et al.*, 1987). Mucin is a normal constituent of saliva and the mucin-based artificial saliva appears to be more effective and better tolerated than other NaCMC based ones (Sweeney *et al.*, 1997, Duxbury *et al.*, 1989, Gravenmade and Vissink, 1993). Many of the published studies available looked at the impact of saliva substitutes that contain mucin, however these were not licensed for use in Ireland. It would appear also that they have now been discontinued in their country of origin, possibly due to concerns regarding BSE. The presence of NaCMC or xanthan gum increases the viscosity of the product and provides adherence of the product in the oral cavity for a prolonged period of time. However their duration of action is still considered to be too short, with a study by Olsson and Axéll (1991), showing changed friction values for only about 15 min, which was only twice as long as using water. Artificial salivas also contain electrolytes in concentrations similar to normal saliva and fluoride to help tooth remineralization. Usually they contain artificial, non-cariogenic sweeteners e.g. sorbitol and flavourings.

Other than the mucin-containing products there have been few published studies regarding the benefits of artificial salivas. Anecdotal evidence refers to a product called *Oralbalance*™ being a preferred product (Ettinger, 1996), however this has also been discontinued. It has been replaced with a similar product called *Bioxtra*™. However there are some available results from clinical trials on saliva substitutes that show while

many of the products contain a number of shortcomings, there is also a strong desire among many patients to continue using a saliva replacement. A NaCMC based saliva substitute showed no significant advantage over a glycerin and lemon swab, although a significant number of patients preferred both of these options to distilled water (control), (Weisenfeld *et al.*, 1983). A mucin containing spray (*Saliva Orthana*™) showed no statistically significant differences between active spray (mucin containing) and placebo spray (no mucin) and neither spray had any major impact on oral microflora. However the majority of patients in both treatment groups wished to continue using the spray (Sweeney *et al.*, 1997). In another clinical trial of *Saliva Orthana*™ significantly greater relief was offered from xerostomia compared with its base (no mucin) or water and the *Saliva Orthana*™ was significantly better at relieving soreness than water. In terms of preference, *Saliva Orthana*™ was ranked significantly higher than either alternative (Duxbury *et al.*, 1999).

The efficacy of three types of polymer based saliva substitutes was examined. High viscosity *vs.* low viscosity saliva substitutes were compared, with results showing that neither saliva substitute or placebo were truly effective. Patients who preferred the polyacrylic acid based saliva substitute had a lower salivary flow rate than patients who preferred the mucin-containing substitute (Van der Reijden *et al.*, 1996).

1.10 Bioadhesion

1.10.1 Introduction

Bioadhesion is defined as the attachment of synthetic or biological macromolecules to a biological tissue (Peppas and Buri, 1985). The study of bioadhesion has received considerable attention in recent years as bioadhesive polymers can provide excellent vehicles for delivery of pharmaceutical agents to a number of mucosal surfaces in the body e.g. respiratory surfaces, gastrointestinal tract and buccal cavity. Numerous

polymers have been shown to have bioadhesive properties and some of these are listed in Table 1.10.1.

Table 1.10.1 Bioadhesive polymers.

	Substance
1.	Sodium carboxymethylcellulose (NaCMC)
2.	Carbomer
3.	Tragacanth
4.	Sodium alginate
5.	Hydroxyethylcellulose (HEC)
6.	Chitosan
7.	Hyaluronic acid

An ideal polymer for a mucoadhesive drug delivery system should have the following characteristics;

1. The polymer and its degradation products should be non-toxic
2. It should be non-irritant to the mucous membranes
3. It should preferably form a strong covalent bond with the mucin-epithelial cell surfaces
4. It should adhere quickly to moist tissue and should possess some site specificity
5. The cost of the polymer should not be high.

Bioadhesive polymers have been considered for use in many areas of the body over the past decade. The areas for which they have mainly been considered for use include gastrointestinally, bucally, nasally and ocularly.

1.10.2 Gastrointestinal use of bioadhesive polymers

The use of bioadhesives in controlled release delivery systems allows for targeting and localization of the drug at a specific site so enhancing the absorption of the drug. Longer *et al.* (1985) showed prolongation of the gastrointestinal transit and higher bioavailability of chlorothiazide using a polyacrylic acid derivative. Akiyama *et al.* (1995) designed mucoadhesive microspheres (AdMMS), which consisted of a drug and an adhesive polymer e.g. cross-linked polyacrylic acid derivative, dispersed in a spherical matrix of polyglycerol esters of fatty acids. These spheres showed strong adherence to the stomach mucosa in rats and Mongolian gerbils. The drug furosemide and the vitamin riboflavin, which both have narrow absorption windows, both showed enhanced absorption due to prolonged gastrointestinal residence, using these mucoadhesive microspheres (Akiyama *et al.*, 1998). The system was tested also with amoxycillin against *Heliobacter pylori,* as a nonspecific targeting drug delivery system, and showed a more effective clearance of *H. pylori* than was seen with amoxycillin suspension (Nagahara *et al.*, 1998).

1.10.3 Nasal use of bioadhesive polymers

Bioadhesive delivery systems for the nasal passage can be in the form of liquid or powder systems. The nasal passage is of particular interest for the delivery of drugs that require a rapid onset of action, and has been successful for relatively lipophilic, low weight molecular drugs e.g. sumatriptan (*Imigran™).* However for more polar and larger hydrophilic drugs, such as peptides and proteins, absorption has been considerably less efficient. This is due to the low degree of absorption as a result of the nature of the molecules and also because of the rapid movement away from the absorption site in the nasal cavity due to the mucociliary clearance mechanism. In order to overcome this problem bioadhesive polymers have been utilized. Morimoto *et al.* (1985) improved the nasal bioavailability of eel calcitonin and insulin using *Carbopol™ 941.* Rydén and Edman (1992) gained a 11% decrease in plasma glucose levels in rats using insulin in

combination with 0.5% polyacrylic acid when compared to the control. Critchley *et al.* (1994) observed an increase in bioavailability from 15% to 77% in rats, for desmopressin in 2% *Carbopol*™ 934 gel solution, compared to a simple solution of desmopressin administered nasally. Bioadhesive powder systems using cellulose, hydroxy propylcellulose and *Carbopol*™ 934, in combination with freeze-dried insulin have been examined by Nagai *et al.* (1984). All formulations gave significant decreases in plasma glucose levels when administered nasally to dogs and rabbits. The crystalline cellulose blended with freeze-dried insulin resulted in a fast decline in the glucose value to 49% of the control value. The most effective formulation developed was where the *Carbopol*™ was freeze-dried with the insulin and then added to the cellulose. However when given to human volunteers, it was found to give quite variable glucose levels.

1.10.4 Buccal use of bioadhesive polymers

The use of bioadhesives in the buccal cavity is two fold. Firstly the bioadhesives can be used in the form of a buccal patch to deliver drugs systemically through the oral mucosa. The oral cavity is a desirable site for systemic drug delivery as it has a rich blood supply that drains directly into the jugular vein, therefore preventing first-pass metabolism. Alur *et al.* (1999) examined the ability of a buccal tablet containing a novel, natural mucoadhesive agent, *Hakea gibbosa,* to offer trans-mucosal sustained delivery of chlorpheniramine maleate. The tablets were shown to offer sustained and controlled release of chlorpheniramine maleate and to maintain an elevated plasma drug concentration during the entire application period.

However a bioadhesive system can also be used to treat local conditions of the oral cavity (e.g. oral Candidiasis, periodontitis) in the form of a patch, gel or tablet. Codd and Deasy (1998) developed a bi-layered tablet with a bioadhesive backing layer consisting of *Carbopol*™ 980 5% and DDWMS 95%, and an upper layer containing miconazole nitrate and chlorhexidine acetate. This formulation resulted in higher salivary levels and a more sustained release of miconazole nitrate when compared to the

proprietary product *Daktarin*™ gel. Salivary concentrations of the gel were very high initially, but fell below the MIC of miconazole nitrate against *C. albicans* after 40 min. approx. Salivary levels of active from the bioadhesive tablet exceeded the MIC for 3½ hr.

1.10.5 Ocular use of bioadhesive polymers

The ocular bioavailability of drugs in conventional eye drops is low (2-10%), due to a large number of problems with the eye including the small area for absorption, the short time of contact, the lipophilicity of the corneal epithelium and tear production among others (Saettone *et al.*, 1999). Therefore bioadhesion has been of interest with helping to increase the bioavailability of drugs in the eye. Saettone *et al.* (1989) investigated a series of vehicles (solutions, hydrogels and solid matrices) prepared with different polymers, four types of poly(acrylic acid) and three different hyaluronic acid derivatives. The hyaluronic acid vehicles containing pilocarpine or tropicamide showed 2.9 and 3.7 fold increases in the miotic and mydriatic AUC values in rabbits when compared with aqueous solutions of pilocarpine and tropicamide. Durrani *et al.* (1995) evaluated the influence of *Carbopol*™ coating on *in vitro* release and *in vivo* bioavailability of pilocarpine coated reverse phase evaporation vesicles. The presence of the *Carbopol*™ coating reduced the drug release *in vitro*, while *in vivo* the coated vesicles showed a significantly prolonged duration of action compared with uncoated ones.

1.10.6 Mechanisms of bioadhesion

The mechanisms of bioadhesion are numerous and not completely understood. Bioadhesive delivery systems may bond with a cell layer, a mucous layer or a combination of the two. For bioadhesion to occur a series of steps must take place. There must be firstly intimate molecular contact made between the bioadhesive and the mucin/epithelial layer. This is followed by entanglement between the mucin and the polymer chains. Finally primary covalent bonds or secondary non-covalent bonds will

form between the molecules (adsorption). The theories relating to the mechanisms of bioadhesion are as follows,

(a) Wetting theory

This was developed predominantly in regard to liquid adhesives and uses interfacial tension to predict spreading and in turn adhesion. The theory was developed by Peppas and Buri (1985) and while the practical application has been limited it has been examined by Lehr *et al.* (1992). Contact angle measurements can be used to characterize the hydrophilicity/hydrophobicity of surfaces. This was used to determine the hydrophilicity/hydrophobicity of polycarbophil (fully hydrated) and pig intestinal mucosa, using the captive bubble technique. Three test fluids, isotonic saline (IS), artificial gastric fluid (GF) and artificial intestinal fluid (IF) were examined. It was found that the hydrated polycarbophils were relatively hydrophilic, indicated by small water contact angles, 22° (IS), 23° (GF) and 16° (IF). However the water contact angles measured with the mucosal tissue were significantly larger, showing that the mucus had a certain degree of hydrophobicity, 61° (IS), 48° (IF), 57° (GF). When force of detachment was measured between the polycarbophil and pig intestinal mucosa, it was found to be largest in IS, intermediate in GF and minimal in IF. When the difference in surface polarities was examined it was seen that there was agreement between the two sets of data.

(b) Diffusion theory

This is the concept that interpenetration and entanglement of bioadhesive polymer chains and mucous polymer chains produce semipermanent adhesive bonds. Jabbari *et al.* (1993) gave supporting evidence to this theory using attenuated total reflection Fourier transform infrared (ATR-FTIR). A thin cross-linked film of polyacrylic acid was formed on an ATR crystal. A mucin solution was placed in contact with this film and the spectra collected over a period of time. A peak after 6 min at 1550 cm^{-1} was attributed to mucin dimeric carboxylic C=O stretching, and it was proposed that this indicated the presence of interpenetrating molecules. It was thought that bond strength increased with

the degree of penetration of the polymer chains into the mucous layer. For interpenetration and entanglement to occur, the bioadhesive molecule and the mucin network must first be brought into intimate contact. The level of penetration and entanglement will then be dependent on concentration gradients and diffusion coefficients. Diffusion coefficient is dependent on molecular weight and the importance of this factor is seen in a study performed by Smart *et al.* (1984), who on examining different viscosity grades of sodium carboxymethylcellulose found that optimum adhesion occurred with molecular weights greater than 78,600 Da. It is thought that interpenetration in the region of 0.2-0.5 μm is required to produce an effective bond. Cross-linking of components will tend to hinder interpenetration, reducing the effectiveness of this mechanism of bioadhesion.

(c) Adsorption theory

Adsorption may occur through the formation of primary 'covalent bonds' or secondary 'non-covalent bonds'. Primary 'covalent bonds' form with proteins on epithelial cells and will be very strong. However the presence of a mucus layer may inhibit direct contact between the bioadhesive polymer and the substrate thus preventing the formation of these covalent bonds.

Secondary chemical bonds consist of hydrogen bonds, van der Waals interactions and ionic bonds. Although individually these bonds are weak, strong adhesions can be produced through having numerous sites of bonding. This means that polymers with high molecular weights and large numbers of polar groups e.g. –COOH and –OH, tend to be good mucoadhesive agents.

1.10.7 Factors important for bioadhesion

(a) Molecular weight

Bioadhesion in many cases increases up to a certain molecular weight, after which there will be little increase. This molecular weight is dependent on the size and configuration of the polymer molecule e.g. with polyethylene oxide, adhesive strength increases up to molecular weights of 4,000,000, as these polymers have a highly linear configuration. However dextran molecules do not exhibit increased adhesion in molecules with a molecular weight greater than 200,000 (Dûchene *et al.*, 1988). *Carbopol*™ was shown to have increasing bioadhesion with molecular weights from 450,000 to 750,000, followed by a progressive decrease at higher molecular weights (Dûchene and Ponchel, 1997).

Polymer conformation must also be considered. Poly(acrylic acid) molecules exist in a coiled form when unionized, as a result of intramolecular hydrogen bonds between the unionized carboxylic acid groups. When the polymer is ionized, the polymers uncoil to a certain extent, allowing better penetration of the mucin network (Hassan and Gallo, 1990).

(b) Concentration of active polymer

High concentrations e.g. 100% poly(acrylic acid), have been shown to reduce bioadhesion compared to lower polymer concentrations, due to the fact that at lower concentrations polymer chains are looser and have more space to diffuse into the mucus (Peppas and Buri, 1985). Gurny *et al.* (1984) showed increasing bioadhesion with NaCMC up to concentrations of 20%, thereafter it was seen to decrease. At higher concentrations, bioadhesion decreases, either because of shielding of the active groups or macromolecular slippage. It has also been shown (Ranga Rao and Buri, 1989), however, that a minimum number of chains are required for bioadhesion to occur as when in situ bioadhesion tests were performed on polymer microparticles coated with 2% cellulose ether they did not bind to mucus.

(c) polymer chain flexibility

Flexibility is important for interpenetration and chain entanglement. De Vries and Boddé (1998) stated that in order to obtain a mucoadhesive hydrogel, molecular mobility of the polymer groups must be optimized. As polymers become cross-linked, the mobility of individual polymer chains decreases. The effective length of the chain, which can penetrate into the mucus layer, is reduced and mucoadhesive strength is less.

(d) pH

pH will influence the charge on both the surface of mucus and certain polymers. Mucus will have a different charge density, depending on pH because of differences in dissociation of functional groups on the carbohydrate moiety and amino acids of the polypeptide backbone. Polymers will also be affected by pH with polyacrylic acid being an excellent example. As the tightly coiled molecule uncoils at pH 5-7, this allows for greater entanglement and interpenetration resulting in improved bioadhesion, while at lower or higher pH there is decreased bioadhesion. Chary *et al.* (1999) saw maximum adhesion between pH 5-6 for HPMC, with a decrease in bioadhesion at either higher or lower pH.

1.11 Fluoride

1.11.1 Introduction

Fluoride plays two main roles in the oral cavity

1. Prevention of the formation of dental caries
2. Arrest of caries lesion progression

Fluoride concentrations in plaque are related to concentration and exposure to the ion through water, food and dental products. Fluoride is only found in high concentrations in two dietary foods, tea and fish. In fish it is mainly contained in the bones and skin so it is only of relevance in fish such as sardines, where the whole fish is eaten.

In order to obtain substantial protection, administration must be consistent and continuous throughout the period of tooth development. In many places throughout the world, including Ireland, fluoride is added to the municipal supply of drinking water. The optimal concentration of fluoride ion in drinking water is 1 part per million (ppm). However in areas where fluoride is not added to the municipal water supply fluoride supplements are necessary to provide protection against dental caries. The American Dental Association (ADA) and the American Academy of Paediatrics (AAP) currently state that in areas where fluoride supplementation is necessary, children should receive fluoride supplements daily from 6 months to 16 years to provide maximum benefit. The dosing regimen currently recommended is shown in Table 1.11.1.

Table 1.11.1 Recommended doses of fluoride.

Childs age	*Dosage regimen*	*Fluoride in water*
6 months – 3 years	0.25 mg/daily	≤ 0.3 mg/ L
3 years – 6 years	0.50 mg/daily	≤ 0.3 mg/L
3 years – 6 years	0.25 mg/daily	0.3 – 0.6 mg/L
6 years – 16 years	1.0 mg/daily	≤ 0.3 mg/L
6 years – 16 years	0.50 mg/daily	0.3 – 0.6 mg/L

1.11.2 Fluoride and enamel

Flouride can be deposited at three stages of enamel development. At the beginning of enamel formation the enamel forming cells (ameloblasts) lay down a partially mineralized protein matrix. This contains low levels of fluoride, which is taken from tissue fluids and incorporated into apatite crystals. This enamel is porous and readily accumulates ions and other molecules, and it has been shown that fluoride is preferentially absorbed at this stage. A fluoride peak is usually found at or just before the enamel begins to mineralize rapidly i.e. at the maturation stage. Fluoride is not homogeneously distributed across the thickness of enamel, being thickest at the tooth surface and lowest at the interior.

Acquisition of fluoride by the enamel surface appears to continue while the tissue remains porous. Once the enamel is fully mineralized, penetration of fluoride is very slow and normally fluoride does not penetrate this enamel to any appreciable extent. However if the enamel becomes porous due to demineralization or chemical destruction of the apatite lattice, fluoride uptake is increased.

The importance of fluoride in the early developmental stages of teeth has been demonstrated *in vivo* extensively. Ripa *et al.* (1983) examined the effect of weekly rinsing with a 0.2% neutral NaFl solution on caries prevalence of the permanent dentition over 5 years. First to sixth grade children who rinsed from one to five years respectively had a caries prevalence reduction of 45.3% and 47.4% compared to baseline scores of children who were examined before rinsing began. Two variables influenced the cariostatic benefits associated with fluoride rinsing, the younger the children entered the program and the longer they rinsed the greater the accumulated benefits. Zimmer *et al.* (1999) evaluated the effectiveness of application of a fluoride varnish to children in socially deprived area, at high risk of dental caries. The study showed that children who received a minimum of two applications of fluoride varnish per year showed a significantly lower caries increment in comparison to the control group.

1.11.3 Caries formation

Once enamel has erupted into the oral cavity, its surface constantly undergoes modification. After tooth eruption the cariostatic effect of fluoride on teeth in children and adults results from combined effects on bacterial metabolism in plaque, and on the dynamics of enamel demineralization and remineralization during acidogenic challenges. Soon after eruption the enamel surface will become covered to varying degrees by microbial deposits, which when clinically detectable become known as plaque. These microbial deposits will have continuous metabolic activity occurring, resulting in periods of demineralization and redeposition of mineral, dependent on fluctuations in pH at the

surface. A decrease in the pH of the oral fluids will cause a dramatic increase in the solubility of the enamel apatites. The more fluoride the apatite contains the less soluble it will be at the same pH compared to pure hydroxyapatite. The early signs of caries destruction appear as dissolution of the enamel crystals at the very outer surface, with the porosity of the surface increasing significantly. This allows more extensive diffusion of acid into the enamel and results in diffusion out from the enamel of calcium and phosphate ions. This over time results in the formation of a caries lesion. Further enamel dissolution may progress resulting in the entire enamel layer being affected, with the dentin even being reached. Once the enamel surface breaks down a true cavity starts to form and acidic microorganisms will start to occupy this cavity (Fejerskov and Clarkson, 1996).

1.11.4 Arrest of caries lesion progression

Caries lesion development may be arrested at any stage by removal of the causative agent i.e. the microbial deposit from the diseased site. The fact that saliva is supersaturated with respect to fluorapatite and hydroxyapatite, it would be expected that the hard tissues would regain mineralization by deposition. Very early enamel caries lesions may regress and even disappear when the cariogenic challenge is removed. However even though it may appear to be gone, the dissolution of enamel may have penetrated through to the dentin, and there will be a certain degree of porosity remaining. Flouride helps with this problem due to its effects on the redeposition of calcium and phosphate in demineralized areas. Fluoride is said to accelerate the process of remineralization as well as affecting the quality, quantity and position of reprecipitated material. From *in vitro* experiments performed, the following functions of fluoride in arresting caries lesions progress have been determined:

- At very low concentrations when in solution, fluoride initiates mineral phase transformations e.g. dicalcium phosphate dihydrate into fluoroapatite in the lesion, thus rendering the mineral less susceptible to further acid dissolution

- Fluoride may foster the production of larger crystals, again resulting in them being less susceptible to acid dissolution
- Depending on the calcium and phosphorous concentrations in the remineralizing solutions, fluoride may help deposit mineral preferentially in zones of the sub-surface lesion (Fejerskov and Clarkson, 1996).

Numerous studies have been performed which support the ability of fluoride to arrest caries development. Baysan *et al.* (2001) showed that a dentrifice containing 5000 ppm fluoride gave a significant result in the reversal of primary root caries, with 56.9% of the subjects showing one or more primary root caries lesions becoming hard at 6 months. Cain *et al.* (1994) examined dose related remineralization of enamel lesions *in situ*, using five regimens. Increased remineralization was seen with increases in salivary fluoride and there was a clear dose related response. Alexander and Ripa, (2000) compared the effectiveness of three different fluoride regimens on the demineralization that often occurs due to orthodontic treatment. The three regimens examined were, the control group, which used a low potency, high frequency fluoride rinse, group 2 which used a high potency, high frequency fluoride brush on gel and group 3 who used a high potency high frequency, fluoride dentrifice. When pretreatment levels of demineralization were compared to post-treatment values, groups 2 and 3 displayed a significant reduction in smooth surface demineralization sites when compared to the control.

1.11.5 Effect of fluoride on bacterial acid production

The activity of fluoride as an enzyme inhibitor has been recognized for many years and it has been suggested that part of the role fluoride plays in preventing caries is due to the fact that it can cause inhibition of acid production by plaque bacteria. Initial studies showed that concentrations of fluoride required were high, with ten ppm fluoride required for a decisive inhibition. However it was later found that the sensitivity of the bacteria to fluoride was greatly increased at lower pH, at pH 5.0 six ppm fluoride gave complete inhibition of acid production by plaque bacteria. Estimated concentrations of

fluoride in plaque have varied from 5 ppm to 25 ppm, with large inter-subject variability being shown. The main site of fluoride inhibition is the enolase enzyme, which converts phosphoglyceric acid to phosphoenolpyruvic acid (PEP), which prevents the formation of lactic acid. This has several consequences when it occurs in plaque.

- The reduced production of lactate impairs the ability of the bacterial plaque to cause caries.
- In many bacteria the uptake of glucose requires the presence of PEP, so uptake is reduced by the actions of fluoride.
- Some bacteria e.g. *S. mutans*, also take up glucose by another mechanism, 'proton motive force'. The uptake by this mechanism depends on the ability of the microorganism to expel protons, which is controlled by fluoride sensitive enzymes.
- The reduced glucose uptake also prevents the synthesis of glycogen, the intracellular polysaccharide that acts as a store of carbohydrate and makes it possible for oral bacteria to continue to produce acid after the dietary sugar has been washed away by saliva.

(Murray *et al.*, 1991)

1.11.6 Formulations currently available

Supplemental fluoride delivery can be achieved in a number of ways. These include:

- Fluoride supplementation of municipal water supplies
- Dentrifices containing fluoride
- Fluoride gel trays and varnishes
- Fluoride mouthwashes
- Fluoride intraoral releasing devices

The world's first artificial fluoridation plant began operation in Grand Rapids, USA, in 1945. By 1986 over 53% of Americans on piped water supply received fluoridated water. In a national survey of US children, a 39% lower caries prevalence was found in 5 yr olds continuously resident in optimally fluoridated communities compared with

those living in areas lacking fluoride treatment. In Ireland fluoridation became mandatory in 1960 and by 1987 60% of the population were receiving fluoridated water (Murray *et al.*, 1991). O'Mullane *et al.*, (1988) performed a national survey of children's dental health in Ireland post fluoridation. In the case of 5 yr olds who had been lifetime residents of fluoridated communities, 52% were free of caries compared to 38% in the non-fluoride group.

The localized delivery of fluoride to the oral cavity is achieved through the use of mouthwashes, dentrifices, gel trays and intraoral releasing devices. The concentration of fluoride contained in these applications vary greatly, ranging from 225 ppm in 0.05% sodium fluoride mouthrinse to almost 20,000 ppm in an 8% stannous fluoride application. In general high fluoride concentration agents are applied professionally and low fluoride concentration agents are applied by the individual (Horowitz and Ismail, 1996). Some of these preparations provide sustained release over prolonged periods of time e.g. fluoride varnishes, intraoral fluoride releasing devices. Other products e.g. dentifrices and mouthwashes only provide a short period of increased fluoride levels.

Professionally applied fluoride solutions include, neutral sodium fluoride solution, stannous fluoride, acidulated phosphate fluoride, fluoride gels, and fluoride varnishes. Neutral sodium fluoride solution was first used in the USA in the 1940's, however the frequency of application (every 3 months) meant that it was unsuitable for long-term use, although its anti-caries efficacy was proven. Stannous fluoride treatments are required every 6 months. Stannous fluoride is relatively unstable so a fresh solution is required for each patient. Studies have shown a reduction in dental caries of just over 30% with the use of 8% stannous fluoride in unfluoridated communities. Acidulated phosphate fluoride was developed on the premise that greater fluoride uptake occurs under acidic conditions, however it has been shown to be as beneficial only as other fluoride applications.

The fluoride gel tray was developed, as it was apparent that the high viscosity of the gels would make them easier to work with and would allow the entire dentition to be treated at once. The tray is filled with the fluoride gel and placed over the teeth for a defined period of time. For professional application this method is useful, but for personal use it is messy and compliance is poor (Meyerowitz and Watson, 1998). Fluoride varnishes are concentrated solutions of fluorides that when applied to the tooth surface, dry out leaving a layer of fluoride rich varnish attached to the tooth. There are a number of different application frequencies associated with them depending on the caries risk. They have been proven to be effective anti-caries agents, offering a 40–55% reduction in caries incidence when used correctly. Fluoride varnishes were originally developed in Europe and until recently had not been approved for use in the US. In 1994 the FDA approved their use as cavity liners and desensitizing agents, but they are still not approved for use as preventative agents. The problem with both fluoride varnishes and gels is that due to the high concentrations being applied there is the risk of fluoride toxicity if there is accidental ingestion following application (Vaikuntam, 2000).

Intraoral fluoride releasing (IFR) devices are membrane-reservoir type systems formed by an internal element (the matrix) containing granulated sodium fluoride in an external housing (the membrane fluoride retainer). These devices are manufactured with copolymers, which absorb a defined amount of water leading to the release of a constant rate of fluoride (Marini *et al.*, 1999). They can be used to release fluoride for prolonged periods of time of up to 12 months. The main problems posed by them are discomfort, irritation, the fluoride matrix falling out of the retainer and the fact that they have to be fitted by a dentist. Davidson *et al.* (1985) examined the durability of an IFR device in 40 children aged 12-18 years of age, with only 8 of the children retaining the original device for the duration of the 6 month study. However Billings *et al.* (1998) showed 85% retention of the devices over 6 months. It is obvious that the method of application and the area to which the IFR device is applied are crucial. Meyerowitz and Watson (1998) examined the use of an IFR device in patients receiving radiotherapy of the head and neck, as an alternative to a sodium fluoride gel tray. It was found that patients preferred

the presence of the IFR device to the inconvenience of using a gel tray every day. The authors were also looking at ways of simplifying fluoride tablet replacement and smooth the contours of the device to reduce irritation. IFR devices have also been used to prevent caries formation in patients receiving orthodontic treatment. Studies have shown that between 50 and 75% of patients develop demineralization of the buccal surfaces during fixed appliance therapy (Gorelick *et al.*, 1982). Marini *et al.* (1999) showed an increase in salivary fluoride levels from 0.05 µg/ml (pre-treatment) to 0.46 µg/ml during the treatment phase. The IRF devices released fluoride continuously over time, 24 hours a day over 6 months. This method appeared to avoid the development of white spot lesions and to prevent areas of demineralization. Also as the dose being released is low there is no risk of fluorosis or fluoride toxicity.

Bottenberg *et al.* (1991) developed bioadhesive oral tablets containing sodium fluoride. The bioadhesive polymers examined included modified starch, polyacrylic acid, polyethylene glycol and sodium carboxymethylcellulose. Polyacrylic acid showed the best bioadhesion during *in vitro* testing. Dissolution testing revealed that the tablets released all of their fluoride within 8 hr, with between 20% and 50% being released within the first hour depending on the formulation, with the polyacrylic acid containing formulation showing the fastest release. *In vivo* fluoride levels were sustained significantly longer than those obtained with fluoride toothpaste, however there was a high level of fluoride variation *in vivo*.

Fluoride dentifrices and fluoride mouthwashes are easy to use and can be applied on a continuous basis with little supervision. They are a useful way of delivering fluoride during the period of tooth formation and during adulthood, and their impact in the prevention of caries has been well documented. Ripa *et al.* (1983) showed a reduction in caries prevalence using a 0.2% sodium fluoride solution as a mouthwash over a five-year period. However the length of time fluoride is actually present in the oral cavity is short lived. As a result they are not suitable for people suffering a cariogenic challenge due to orthodontic treatment, radiation treatment or systemic disease, where higher

concentrations for more prolonged periods are required. Their main advantage is their ease of use and the fact that no professional attendance is required.

1.11.7 Toxicology

Fluoride is a toxic compound with most cases of acute fluoride poisoning having occurred as a result of accidental ingestion of insecticides or rodenticides containing fluoride salts. Symptoms of acute fluoride overdosage include hypersalivation, epigastric pain, nausea and vomiting, diarrhea, muscle weakness and tremors.

Prolonged daily ingestion of excessive amounts of fluoride during the period of tooth development may result in dental fluorosis. Dental fluorosis is characterized by the appearance of mottled enamel due to hypocalcification with the extent of damage dose dependent. The effect of fluorosis is generally considered more cosmetic than functional. The principal adverse functional effect of excess fluoride intake is skeletal fluorosis, which manifests itself in three stages. Stage 1 is characterized by occasional joint stiffness and pain, along with some pelvic and vertebral osteosclerosis. In stages 2 and 3 calcification of ligaments, osteosclerosis, possible osteoporosis of long bones, muscle wasting and neurological defects occur.

Chapter 2

Materials

Description	**Manufacturer/Supplier**
Acetonitrile HPLC grade	Lab Scan - various lots
Agar No. 1	Oxoid Lot no. 811967
Brain heart broth	Oxoid Lot no. 225433
Calcium chloride	Merck Lot no. TA 448382 831
Carbopol 974P™	BF Goodrich B/N AB006N3
CDTA (trans-1, 2-Diaminocyclohexane-N, N, N', N'-tetraacetic acid)	Sigma Lot no. 108H2611
Chitosan (medium M_w)	Aldrich Lot no. 06126MN
Citric acid (monohydrate)	Merck - various lots
Compritol 888 ATO™	Gattefosse Lot no. 23636
Cottonseed oil	Sigma Lot no. 149L3721
Drum dried waxy maize starch	Cerestar n.v.
Ethylenediaminetetraacetic acid	Sigma Lot no. 68H0125
Glacial acetic acid	Sigma Lot no. 40K3474
Glucose monohydrate	Riedel-de Haen Lot no. 02750
Halloysite 'G'	New Zealand China Clays Ltd. B/N 12/98
Lemon flavouring	Dragoco 9/021145
Magnesium chloride	BDH Lot no. 290964Y
Octyl cyanoacrylate	Henkel Lot no. 2395/71
Phenylmethylsulphonylfluoride	Sigma Lot no. 88H0793
Pluronic F68™	BASF Lot no. WDPH-521
Poloxamer 407	BASF Lot no. 57-0119
Polyethylene glycol 6,000	Fluka - various lots
Polyethylene glycol 20,000	Fluka Lot no. 81275
Porcine gastric mucin (Type II – Crude)	Sigma Lot no. 48H0596
Potassium chloride	Sigma Lot no. K25691637
Potassium hydroxide	Sigma Lot no. 110K0244
Potassium nitrate	BDH Lot no. 29638

Potassium phosphate (monobasic)	Sigma Lot no. 117H0061
Precirol ATO5™	Gattefosse Lot no. 23707
Sodium acetate (trihydrate)	BDH Lot no. 10235
Sodium acid phosphate	Merck Lot no. K91134545
Sodium azide	Sigma Lot. no. 334S7184
Sodium bicarbonate	Merck - various lots
Sodium chloride	Merck Lot no. K26025300 905
Sodium fluoride	Sigma Lot no. 229B432341
Sodium lauryl sulphate	Sigma Lot no. 39H0077
Sodium phosphate	Merck - various lots
Sorbitol	Sigma Lot no. 108H01461
Sunflower oil	Tesco pure sunflower oil 0278 HA
Tetracycline base	Sigma Lot no. 78H0745 and 110K1491
Tetracycline hydrochloride	Sigma Lot no. 89H0915
Triclosan 5000	Kumar Organic Products B/N K8TC5-5000-OC6R
Xylitol	Lancaster B/N 10014882
Zinc sulphate	Merck Lot no. TA490683 84

Chapter 3

Methods

3.1 Formulation of controlled release drug delivery systems using the novel excipient halloysite

3.1.1 Introduction

The aim was to formulate two different products, with the novel excipient 'halloysite' being used to help provide controlled release of the drugs. Firstly a product for the localized treatment of periodontitis was developed, halloysite was drug loaded with tetracycline base and coated with chitosan, which was then delivered using a thermoresponsive gel system. Secondly a bioadhesive tablet containing sodium fluoride to aid in the prevention of caries development was formulated. The halloysite was drug loaded with sodium fluoride, coated with chitosan and then microencapsulated to form solid lipid microparticles.

3.1.2 Drug loading of halloysite

The halloysite 'G' supplied by New Zealand China Clays Ltd. was first sieved through a 125 μm sieve to remove large aggregates. The sieved halloysite sample and the drug solution were mixed in a 1:1 ratio. The halloysite was loaded with three different drug systems, tetracycline base (20 mg/ml) in ethanol or methanol, tetracycline HCl (20 mg/ml) in ethanol, and sodium fluoride (40 mg/ml) in distilled water. Each of these mixtures was transferred to a freeze-drying flask, which was attached to the condenser of a freeze-drying apparatus (Hetosicc, Denmark), and a vacuum was applied using an Edwards RV5 vacuum pump. After a short period of time, air bubbles were observed appearing on the surface of the mixture, which represents air being drawn out of the hollow tubules. This vacuum was maintained until all the air in the tubules had been apparently displaced. The vacuum was discontinued and the preparation mixed. The vacuum was then reapplied to remove any further trapped air. This procedure was repeated once more. The drug-loaded samples were then placed in a forced air circulation oven at 50°C and dried to a constant weight. The sample was removed from

the oven and lightly ground down using a mortar and pestle, and passed through a 125 μm sieve to break up any large aggregates. The remaining sample was weighed and the drug loading procedure was repeated, using the same 1:1 ratio for drug loading. After drying, the double drug loaded sample was passed first through a 125 μm sieve and then through a 90 μm sieve to produce a fine powder.

3.1.3 Coating drug loaded halloysite with chitosan

A 0.2% w/v solution of chitosan in acetate buffer pH 4.2 was prepared. A suitable quantity of double drug-loaded halloysite was weighed out and placed in a Kenwood chef planetary mixer, with the appropriate volume of the chitosan solution. This was blended at speed 1.0 for a defined period of time (30 s or 90 s) to allow charge neutralization to occur between the halloysite and the chitosan. The preferred binding ratio of halloysite:chitosan is 1:0.114 (Levis, 2000). The suspension was transferred to 50 ml centrifuge tubes and centrifuged at 3,000 rpm for 10 min. The supernatant was discarded and the sediment was dried for 24 hr at 50°C to constant weight. The dried powder sediment was ground lightly with a mortar and pestle and passed firstly through a 125 μm sieve and then through a 90 μm sieve to produce a fine powder.

3.1.4 Preparation of *poloxamer 407* (*Lutrol F127™*) samples

a. Preparation of systems containing *poloxamer 407* and polyethylene glycol (PEG)

Distilled water was placed in a pre-weighed beaker. The required quantity of the particular molecular weight polyethylene glycol (PEG) as shown in Table 3.1.1 was added to the beaker and stirred on a mechanical stirrer until fully in solution. The beaker was transferred to a mechanical stirrer, which was located in a walk-in refrigerator (temperature 4°C), and the temperature was allowed to reach 4°C. The required quantity of *poloxamer 407* as shown in Table 3.1.1 was added to the beaker slowly over

2 hr approx. with constant stirring, and left overnight to hydrate at 4°C. The system was then made up to final weight with distilled water.

b. Preparation of systems containing *poloxamer 407*, PEG and octyl cyanoacrylate (octyl CA)

Distilled water was placed in a pre-weighed beaker. The required quantity of the particular molecular weight PEG (Table 3.1.1) was added to the beaker and stirred on a mechanical stirrer until fully dissolved. The beaker was transferred to a mechanical stirrer, which was located in a walk-in refrigerator (temperature 4°C), and allowed to reach 4°C. The required quantity of *poloxamer 407* as shown in Table 3.1.1 was added to the beaker slowly over 2 hr approx. with constant stirring and left overnight to hydrate at 4°C. The pH of the solution was adjusted to 4.0 ± 0.05 using a 10% v/v aqueous solution of glacial acetic acid. The beaker was transferred back to the refrigerator and the required quantity of octyl CA (Table 3.1.1) was added in small increments with continuous stirring. Finally, the preparation was made up to its final weight with distilled water, and the pH checked to be within the range 4.0 ± 0.05.

3.1.5 Addition of halloysite to *poloxamer 407* systems

In order to minimize the wastage of drug-loaded product, sieved unloaded halloysite was used initially to give a rough determination of the amount of halloysite that was to be used in the final system. The quantity of halloysite chosen (100 mg/ml or 200 mg/ml) was weighed out and placed in a 30 ml glass jar. A quantity of the *poloxamer 407* system was added sufficient to produce a thick paste. The remainder of the *poloxamer 407* system was added in small volumes with constant stirring. This final gel system was filled into 1 ml syringes, each fitted with a 26 gauge blunt tipped needle.

Table 3.1.1 Formulation of *poloxamer 407* systems.

Sample no.	***Poloxamer 407 % w/w***	***PEG 6,000 % w/w***	***PEG 20,000 % w/w***	***Octyl CA % w/w***	***mg halloysite /ml of poloxamer 407 system***	***pH of product***
1	25	////////////	////////////	////////////	////////////	////////////
2	20	////////////	////////////	////////////	////////////	////////////
3	25	5	////////////	////////////	////////////	////////////
4	25	2.5	////////////	////////////	////////////	////////////
5	25	1	////////////	////////////	////////////	////////////
6	20	2.5	////////////	////////////	////////////	////////////
7	20	3.0	////////////	////////////	////////////	////////////
8	20	////////////	0.5	////////////	////////////	////////////
9	20	////////////	1.0	////////////	////////////	////////////
10	20	////////////	1.0	////////////	////////////	4
11	20	////////////	1.0	////////////	200	////////////
12	20	////////////	1.0	////////////	100	////////////
13	20	////////////	1.0	0.5	////////////	4
14	25	////////////	////////////	1.0	////////////	4
15	20	////////////	////////////	1.0	////////////	4
16	20	////////////	0.5	1.0	////////////	4
17	25	////////////	1.0	1.0	////////////	4
18	20	////////////	1.0	0.5	200	4
19	20	////////////	////////////	1.0	200	4
20	20	////////////	0.5	1.0	200	4

3.1.6 Preparation of solid lipid particles containing sodium fluoride

Solid lipid particles were prepared using the 'cold' dispersion technique (Müller *et al.*, 1995). The lipid (*Precirol AT05™* or *Compritol 888 ATO™*) was melted and four systems prepared using the melted lipid. Two of the systems consisted of double drug loaded (4% w/v sodium fluoride) halloysite, coated with chitosan then dispersed in each lipid in a 50:50 ratio of halloysite:lipid. The final two systems consisted of the drug alone (sodium fluoride) dispersed in each lipid in a ratio of 50:50 (sodium fluoride:lipid). The lipid systems were cooled rapidly using liquid nitrogen, resulting in the sodium fluoride loaded halloysite or sodium fluoride alone becoming entrapped in the frozen lipid. The frozen lipid particles were ground down using a mortar and pestle, with liquid nitrogen being added at intervals to maintain the lipid in a brittle state. The ground lipid was finally sieved through a 600 μm sieve. This lipid powder was dispersed at a concentration of 30%, in an aqueous surfactant solution containing 5% w/v surfactant (*Pluronic F68™*) and 5% w/v cryoprotectant (glucose). This dispersion was emulsified using an Ultra Turrax T25 shear mixer at a rate of 7,500 rpm for 5 min, which reduced the size of the solid lipid particles. During emulsification the temperature of the systems was kept at 4°C approx. by keeping the systems immersed in ice. The solid lipid dispersion was frozen in a 100 ml round bottomed flask by immersing and rotating in a chilled propylene glycol bath (Hetofrig, Denmark) for 30 min. The samples were then freeze-dried for 24 hr at maximum vacuum using a Hetosicc (Denmark) condenser attached to an Edwards RV5 pump. The dried powder obtained was ground down and sieved through a 200 μm sieve.

3.1.7 Preparation of bilayered bioadhesive discs containing sodium fluoride solid lipid particles

Two fluoride systems were chosen for compression:

System A: Halloysite double drug loaded with sodium fluoride (4% w/v) and coated with chitosan, entrapped in a solid lipid particle (*Compritol 888 ATO*™), in a 50:50 ratio of halloysite:lipid.

System B: Halloysite double drug loaded with sodium fluoride (4% w/v) and coated with chitosan, entrapped in a solid lipid particle (*Precirol AT05*™), in a 50:50 ratio of halloysite:lipid.

Two-layered bioadhesive tablets were manufactured. The tablets were compressed on a Manesty hand tablet press using 8.5 mm diameter tooling, with flat-faced upper and lower punches. The lower bioadhesive layer, consisting of 20 mg of a physical mixture of drum dried waxy maize starch (DDWMS) 95% w/w and *Carbopol 974P*™ 5% w/w, was placed in the die cavity and lightly pressed. 150 mg of the fluoride system (A or B) was placed in the die cavity on top of the lower bioadhesive layer and the composite was fully compressed.

After compression, the discs from each system were annealed by placing them in an oven at a temperature 5-8°C greater than the melting point of the respective lipids for a period of 10 min. The melting range of *Compritol 888 ATO*™ is 69-74°C and for *Precirol AT05*™ it is 53-57°C.

3.1.8 Scanning electron microscopy (SEM)

SEM was carried out on halloysite 'G', drug loaded halloysite and solid lipid particles containing sodium fluoride. The samples were coated with a thin film of gold in a Polaron SC500 sputter coater (UK). The samples were then examined using a LEO Stereoscan S–360 scanning electron microscope (UK). The analysis was used to determine the surface appearance of the samples.

3.2 Syringeability of *poloxamer 407* systems

The ease with which the *poloxamer 407* systems could be expressed from their syringes and through their fitted needles, was measured using a texture analyzer XT.RA (Stable Micro Systems, UK) in compression mode (Figure 3.2.1). A fully filled 1 ml syringe was held in place with a clamp and the upper probe of the texture analyzer moved downwards until it came in contact with the syringe base. A constant force of 0.5 N was applied to the syringe and the work required to expel the contents for a piston depth of 30 mm was measured. The area under the resulting curve was used to determine the work of expulsion, a measure of the work required to expel part of the contents of the syringe. A number of systems (Table 3.2.1) were examined at 2 different temperatures, 4°C and 20°C, and each system had five replicates performed.

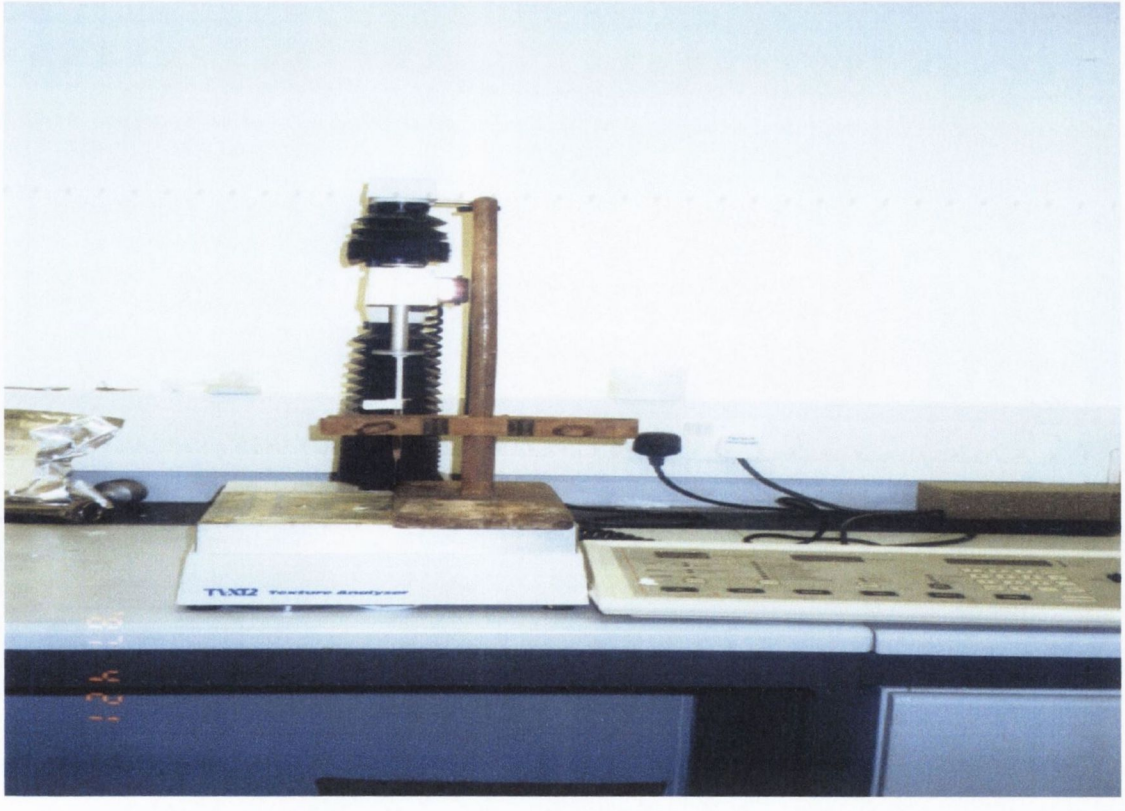


Figure 3.2.1 Apparatus used to test the syringeability of the *poloxamer 407* systems.

Table 3.2.1 *Poloxamer 407* systems examined for syringeability.

1. *Poloxamer 407* 20% w/w
2. *Poloxamer 407* 25% w/w
3. *Poloxamer 407* 20% w/w, PEG 0.5% w/w, water to 100%
4. *Poloxamer 407* 20% w/w, PEG 0.5% w/w, octyl CA 1% w/w, water to 100%, pH 4
5. *Poloxamer 407* 20% w/w, PEG 0.5% w/w, octyl CA 1% w/w, water to 100% and 200 mg/ml halloysite double loaded with tetracycline base and coated with chitosan, pH 4

3.3 Stability study on *poloxamer 407* systems

A stability study on the final formulation (200 mg halloysite double loaded with tetracycline base and coated with chitosan per 1 ml of *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w, water to 100%, pH 4) and the *poloxamer 407* system without the drug loaded halloysite (*poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w and water to 100%, pH 4) was performed over a nine month period with sampling intervals at time 0, 1 month, 3 months, 6 months and 9 months. Samples were stored at two different temperatures, 4°C and 25°C, for the duration of the study.

Five samples of each system at each time point and for each temperature were prepared. At the determined time intervals, 5 samples of each system were taken from the two temperatures they were stored at and tested. The parameters determined are outlined in Table 3.3.1. These parameters were tested using the methods outlined in Section 3.2 (Syringeability of *poloxamer 407* systems) and Section 3.12 (Rheometry). The samples were pooled after syringeability testing and this pooled sample was used for rheological testing to determine gelling temperature and apparent viscosity.

Table 3.3.1 Parameter outlines for stability study of *poloxamer 407* systems.

Visual appearance	***Syringeability (N.mm)***	***Gelling temperature (°C)***	***Apparent viscosity (η_{app})***
Colour and homogeneity	Force of expulsion (N.mm) over 30 mm applying a force of 0.5 N at 20°C.	Oscillatory temperature ramp from 15-45°C over 15 min.	Viscosity temperature ramp from 15-45°C over 15 min at a shear rate of 100 s^{-1}

3.4 Determination of dynamic solubility of tetracycline base or HCl

The dynamic solubility of the antibiotic forms was determined by the following method. Excess sample (3-4 times the approximate solubility) of the drug was added to 50 ml of phosphate buffer pH 6.8 and stirred at a rate of 300 rpm at 37°C. Samples of 5 ml were removed at 5, 15, 30, 60 and 120 min. These samples were filtered through a 0.45 μm membrane filter (Gelman) and diluted as required for analyses. The samples were analyzed in a Shimadzu UV-160 spectrophotometer set at 362 nm. Standard calibration curves for tetracycline base and tetracycline HCl were prepared by obtaining the absorbance for determined concentrations of both drugs at 362 nm. All syringes, needles, filters, pipettes and glassware used in the process were pre-heated in an oven to 37°C and removed immediately prior to use.

3.5 Dissolution studies

3.5.1 Introduction

A non-pharmacoepial dissolution method was used to examine the release of tetracycline from halloysite. This was due to the nature of the physiological system that was being

mimicked i.e. the periodontal pocket, which is a quiescent system with a very small volume. Bioadhesive sodium fluoride tablets were examined using both pharmacoepial and non-pharmacoepial dissolution tests. Again this was due to the fact that the pharmacoepial dissolution test did not mirror *in vivo* conditions adequately. Phosphate buffer pH 6.8 (Pharmaceutical Handbook, 1980) was the dissolution medium used for all dissolution tests performed with tetracycline base, tetracycline hydrochloride and sodium fluoride.

3.5.2 Dissolution testing of halloysite drug loaded with tetracycline base or tetracycline HCl, with and without chitosan coating

For halloysite drug loaded with tetracycline base, and halloysite loaded with tetracycline base / tetracycline HCl then coated with chitosan, 250 mg samples were placed in fine paper mesh bags and tied at the top to ensure the retention of the sample. These were placed in 100 ml Duran flasks containing a defined amount of dissolution medium. The volume of dissolution medium used was dependent on the stage of the dissolution test as it was necessary to achieve sink conditions but also to have detectable levels of drug, and the volumes used are outlined in Table 3.5.1.

Table 3.5.1 Volumes of phosphate buffer pH 6.8 used in dissolution testing of halloysite loaded with tetracycline base / tetracycline HCl and coated with chitosan

Days	***Volume of dissolution medium***
1-29	60 ml
30 - finish	30 ml

The Duran jars were placed in a water bath at 37°C, with samples being taken and the dissolution medium replaced at days 1, 2, 3, 4, 9, 16, 23, 30, 37, 51 and 65. Samples from all dissolution tests (halloysite double drug loaded with tetracycline base and halloysite double drug loaded with tetracycline base or tetracycline HCl then coated with

chitosan) were filtered using a 0.45 μm membrane filter (Gelman). After the last sample was taken, the residue of product was suspended in 100 ml of phosphate buffer pH 6.8 and left to stir for 7 days. Samples of dissolution medium from the residue study were removed and filtered through a 0.45 μm membrane filter (Gelman) prior to UV assay.

3.5.3 Calculations

The absorbance of standard tetracycline base and tetracycline HCl solutions in phosphate buffer pH 6.8 were measured at a wavelength of 362 nm using a Shimadzu UV-160 spectrophotometer. The absorbance values *vs.* their respective concentrations expressed in μg/ml were subjected to linear least squares regression, resulting in a linear calibration equation (Appendix 1). Each unknown sample was measured at a wavelength of 362 nm. The concentration in μg/ml of tetracycline base or tetracycline HCl in solution was calculated for each sample using the relevant calibration equation. The results were presented as percentage drug released.

3.5.4 Dissolution testing of final formulation

When examining the final formulation (200 mg halloysite double loaded with tetracycline base and coated with chitosan per 1 ml of *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w, water to 100%, pH 4), a system more similar to physiological conditions was used. 0.5 ml of the product was injected into the bottom of a 10 ml glass tube and this was placed in a water bath at 37°C for 15 min to allow it to gel. 1.5 ml of dissolution medium (phosphate buffer pH 6.8) was carefully pipetted onto the surface of the product. 1.5 ml was the volume chosen as this is the volume of gingival crevicular fluid produced per pocket in 24 hr (Goodson, 1989). This volume of dissolution medium was removed and replaced by an equivalent amount at days 1, 2, 3, 4, 10, 16, 23, 31, 42 and 63. After the last sample was taken the residue of the product was suspended in 100 ml of dissolution medium and left stirring for one week. The samples

were filtered using a 0.45 μm membrane filter (Gelman) and were analyzed by HPLC (Section 3.6.2).

3.5.5 Dissolution testing of sodium fluoride containing preparations

The dissolution studies for the sodium fluoride containing preparations were performed using an Erweka DT6 six-station dissolution tester. The rotating basket method was used to test the sodium fluoride solid lipid particles in powder form (Section 3.1.6) and the paddle method was used to test the bioadhesive sodium fluoride tablets (Section 3.1.7). The baskets and paddles were rotated at 100 rpm and kept 23 to 27 mm above the base of the vessel (A189, Apparatus 1, British Pharmacopoeia, 2002). The experiments were performed using dissolution media (phosphate buffer) at two pH's: 6.8 and 5.5. 1000 ml of dissolution medium were placed into each of the 6 dissolution vessels. A water bath maintained the dissolution medium at 37°C for the duration of the experiment.

The sodium fluoride solid lipid particle samples were loaded into fine paper mesh bags and sealed at the open end by tying with thread, and were placed into baskets to prevent escape of insoluble contents. At the end of each dissolution test, the mesh bags were removed and placed in an oven at 50°C for 24 hr. The residue was weighed and resuspended in 250 ml of fresh dissolution medium and left to stir on a Minimag stirrer for 24 hr and analyzed to determine the level of sodium fluoride remaining in the powder sample. The bioadhesive bilayered sodium fluoride tablets were attached to the base of the dissolution vessel using octyl CA.

5 ml samples were withdrawn at intervals over 8 hr and were replaced with 5 ml fresh dissolution medium maintained at 37°C. Samples were taken at 5, 10, 15, 30, 60, 120, 180, 240, 300, 360, 420 and 480 min. The sample was taken from a point halfway up the side of the dissolution vessel. The repeated removal of sample from the dissolution medium was accounted for in subsequent calculations. The samples were analyzed using

an Orion fluoride / fluoride combination electrode model no. 96-09. The fluoride probe was attached to an AGB 2000 pH meter that was capable of reading millivolt output.

3.5.6 Dissolution testing of sodium fluoride tablets using a flow through apparatus

The BP dissolution method was not considered adequately representative of *in vivo* conditions and therefore a non-pharmacoepial method was developed. The apparatus used is shown in Figure 3.5.1. A 250 ml conical flask had a glass funnel placed in it. A glass slide was placed sitting into the funnel. A single fluoride tablet (Section 3.1.7) had been previously attached to the glass slide using octyl CA. The top half of a filter housing was placed over the tablet in order to minimize evaporation. Narrow bore tubing was attached to an infusion pump (Perfusor V) containing phosphate buffer pH 6.8. The free end of the tubing was placed in the opening of the filter housing. The whole apparatus was kept in an incubator at 37°C throughout the experiment. A flow rate of 1 ml/min was used. Samples were taken and analyzed as outlined in Section 3.5.7 at 30, 90, 150, 210, 270, 330, 390, 450 and 480 min.

3.5.7 Calculations

TISAB II (Total ionic strength adjustment buffer) was prepared as follows:
glacial acetic acid 5.7% v/v, potassium chloride 7.4% w/v and CDTA 0.1% w/v were mixed together in distilled water until fully dissolved, the pH was then adjusted to 5.5 using 1 M potassium hydroxide and the volume was made up to the mark using distilled water. The millivolt readings of standard sodium fluoride solutions were measured. The millivolt readings *vs.* their respective concentrations, expressed in parts per million (ppm), were plotted on a log scale. This was subjected to linear least squares regression analysis, resulting in a linear calibration equation. A sample calibration curve is shown in Appendix 2. The 5 ml samples taken from the dissolution medium were allowed to cool to room temperature, 5 ml TISAB II was added to the sample and the electrode

was placed in the sample and allowed to settle. The millivolt reading obtained was transformed to ppm using the calibration equation. Calibration curves were prepared on a daily basis, with standards being checked morning and evening for any drift. The electrode was rinsed with distilled water and blotted dry between each measurement.

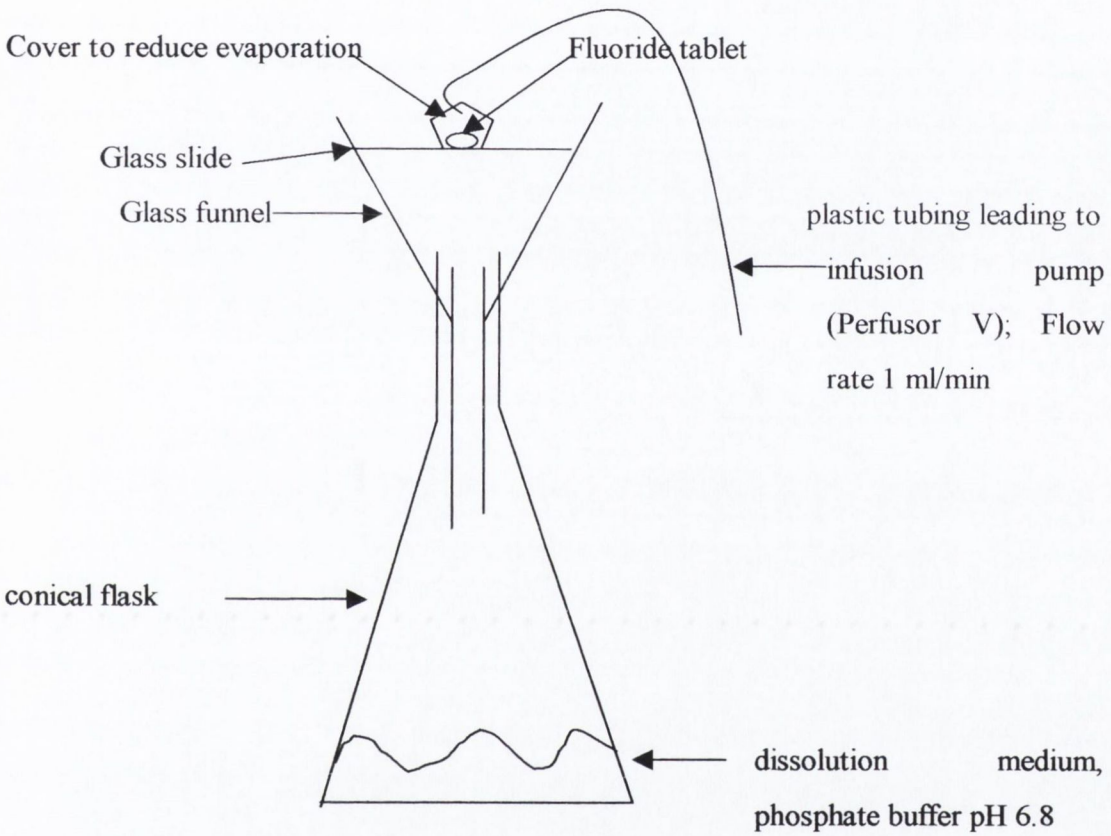


Figure 3.5.1 Diagrammatic representation of flow-through apparatus used for dissolution testing of bioadhesive sodium fluoride tablets.

3.6 High performance liquid chromatography determination of tetracycline base

3.6.1 Introduction

Two methods were used to determine concentrations of tetracycline base present in samples, HPLC using UV detection and HPLC using fluorescent detection. HPLC using fluorescent detection shows sensitivity several orders of magnitude greater than that seen with UV detection. It was necessary to use fluorescent detection due to the extremely low concentrations of tetracycline present in the samples taken from the *in vivo* dog trial. The method used was modified from an assay developed by Vienneau and Kindberg, 1997.

3.6.2 HPLC using UV detection

The tetracycline base concentration in phosphate buffer pH 6.8 samples, obtained from the dissolution test outlined in Section 3.5.4, was determined by reverse phase HPLC using an assay modified from Knox and Jurand, 1979. The samples were diluted using the same buffer in which the dissolution test had been performed. 20 µl samples were injected onto a Thermoquest 25 cm x 4.6 mm column packed with *Hypersil™* 5 µm octadecyl silica. The mobile phase consisted of water:acetonitrile:glacial acetic acid (71:18.5:10.5) v/v/v containing 0.011 M KNO_3 and 2.8 x 10^{-3} M EDTA, with pH adjusted to 3.0 using 0.5 M sodium acetate. Before use the mobile phase was filtered through a Gelman polypropylene 47 mm, 0.2 µm hydrophilic membrane filter and degassed by sonicating. The column was equilibrated with mobile phase until a stable base line was obtained. The flow rate was 1.5 ml/min, pumped by a Shimadzu LC-5A HPLC unit. A Shimadzu SPD-2A variable wavelength UV spectrophotometric detector set at 362 nm and linked to a Shimadzu C-R3A Chromatopac integrator was used for detection of drug. Tetracycline base and tetracycline HCl had retention times of approximately 5.5 and 6.2 min respectively. A sample chromatogram is shown in Appendix 3.

Standard concentrations of tetracycline base and tetracycline HCl were prepared in water:acetonitrile (70:30). The peak areas *vs.* their respective concentrations expressed in µg/ml were subjected to linear least squares regression analysis, resulting in a linear calibration equation. A sample calibration curve is included in Appendix 4. The concentrations of tetracycline base or HCl in µg/ml were calculated for each sample using the calibration equation. Both standards and experimental samples were injected in triplicate.

3.6.3 HPLC using fluorescent detection

The HPLC system comprised a Shimadzu LC-5A HPLC unit equipped with a Shimadzu RF-10Axl fluorescence detector. The fluorescence detector was set with an excitation wavelength of 375 nm and an emission wavelength of 512 nm. The column used was the same as that used in Section 3.6.2 protected by an ODS guard column. The column was preconditioned with the mobile phase until a stable baseline was obtained. The mobile phase consisted of methanol and a 0.1 M sodium acetate buffer containing 25 mM EDTA and 35 mM $CaCl_2$ dihydrate (buffer A, pH 6.5) (40:60, v/v). Before use the mobile phase was filtered through a Gelman polypropylene 47 mm, 0.2 µm hydrophilic membrane filter and sonicated to remove air bubbles.

Doxycycline HCl was used as the internal standard and was included in all standards and clinical samples. The assay was validated for linearity, reproducibility, repeatability and limit of detection. The reproducibility of the assay was determined by examining the r^2 value and slope for the lines of best fit of calibration curves on five consecutive days. The repeatability of the method was determined by running five standards in triplicate on the same day. The limit of detection for tetracycline base was determined by continual reduction of the concentration of tetracycline base until the peak area no longer showed reproducibility or repeatability. The method was also examined for specificity to ensure that there were no interfering peaks at the retention times of interest. Standards were made using the mobile phase and incorporating 200 ng/ml of doxycycline HCl 250 µl

mobile phase (containing 200 ng/ml internal standard) was pipetted into the vials containing the orthodontic points obtained from sampling the pockets for tetracycline residue (Section 3.7.5). These vials were sonicated for 15 min. Triplicate injections of 20 µl were analyzed on the HPLC system and concentrations determined using the calibration curve shown in Appendix 5. A sample chromatograms of tetracycline base and the internal standard, doxycycline HCl is shown in Appendix 6.

3.7 *In vivo* testing of tetracycline formulation

3.7.1 Preparation of samples for use in *in vivo* study

All samples were prepared 72 hr prior to the start of the study. A suitable quantity of halloysite was double drug loaded with tetracycline base using the method described in Section 3.1.2 and this was coated with chitosan using the method described in Section 3.1.3. A *poloxamer 407* system containing *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w and octyl CA 1% w/w, water to 100% w/w was prepared as outlined in Section 3.1.4 (b). The drug loaded halloysite was incorporated into the *poloxamer 407* system at a concentration of 200 mg halloysite /ml of *poloxamer 407* system (test system). Halloysite that had not been drug loaded was coated with chitosan and incorporated into the same *poloxamer 407* system at the same concentration (control system). 24 x 1 ml syringes fitted with 26 gauge blunt tipped needles were weighed individually on a Mettler AE 100 balance, numbered and the weights recorded (Appendix 7). 12 of these syringes were filled with the test system and the other 12 were filled with the control system. The syringes were reweighed and the weights recorded (Appendix 7). Temperature ramps as described in Section 3.12.2 were performed on samples to ensure they were gelling at the correct temperatures. The samples were removed from the cold room on the morning of their use and stored on ice until the time of application. Samples were mixed manually by shaking, prior to injection into periodontal pockets, to ensure a uniform mixture was achieved. After the syringes were reweighed to determine the amount used in the injection process

(Appendix 7) and the remainder was frozen at -20°C to be analyzed subsequently for total tetracycline content.

3.7.2 Determination of total tetracycline content in test samples

100 mg of product from each syringe was weighed and placed in a 10 ml volumetric flask. Each flask was made up to the mark with methanol and left to sonicate for 15 min. Samples were taken from each volumetric and filtered through a 0.45 μm membrane filter. A standard calibration curve of tetracycline base in methanol was prepared using UV detection at 362 nm. The samples were analyzed and the concentration of tetracycline base in each sample determined from the prepared calibration curve.

3.7.3 *In vivo* dog study

Two healthy, mature (2 year old) male beagle dogs were selected for the study. Each dog had three pairs of contralateral maxillary teeth and mandibular teeth (canines and premolars) tested (Table 3.7.1). On canines, a mucoperiosteal flap was raised and pockets were surgically created of 4 mm corono-apically and 6 mm mesio-distally (surgical wound pocket creation model). Furcation defects were surgically created on the UP2, UP4, LP2 and LP4 (Class II furca of 3 mm horizontal and 2 mm vertical) as shown in Figure 3.7.1 and Figure 3.7.2.

Table 3.7.1 Abbreviated terms used to identify sample pockets.

Abbreviated term	***Position in oral cavity***
URP2	Upper right premolar furcation
LRP2	Lower right premolar furcation
LRC	Lower right canine flat surface
LRP4	Lower right premolar furcation
URC	Upper right canine flat surface
URP4	Upper right premolar furcation
ULP2	Upper left premolar furcation
LLP2	Lower left premolar furcation
LLC	Lower left canine flat surface
LLP4	Lower left premolar furcation
ULC	Upper left canine flat surface
ULP4	Upper left premolar furcation

Three pairs of pockets were used as vehicle controls (Table 3.7.2). In the remaining contralateral pockets (Table 3.7.2), the test samples were injected into the bottom of the pocket as shown in Figure 3.7.3. Pockets were sampled at days 14, 28, 42 and 56 for microbiological testing and tetracycline concentrations. All pockets were sampled at each time point.

Table 3.7.2 Distribution of samples for canine teeth.

Sample no.	***Tooth***	***Dog 1***	***Dog 2***
1	URP2	TEST	CONTROL
2	LRP2	TEST	CONTROL
3	LRC	TEST	CONTROL
4	LRP4	TEST	CONTROL
5	URC	TEST	CONTROL
6	URP4	TEST	CONTROL
7	ULP2	CONTROL	TEST
8	LLP2	CONTROL	TEST
9	LLC	CONTROL	TEST
10	LLP4	CONTROL	TEST
11	ULC	CONTROL	TEST
12	ULP4	CONTROL	TEST

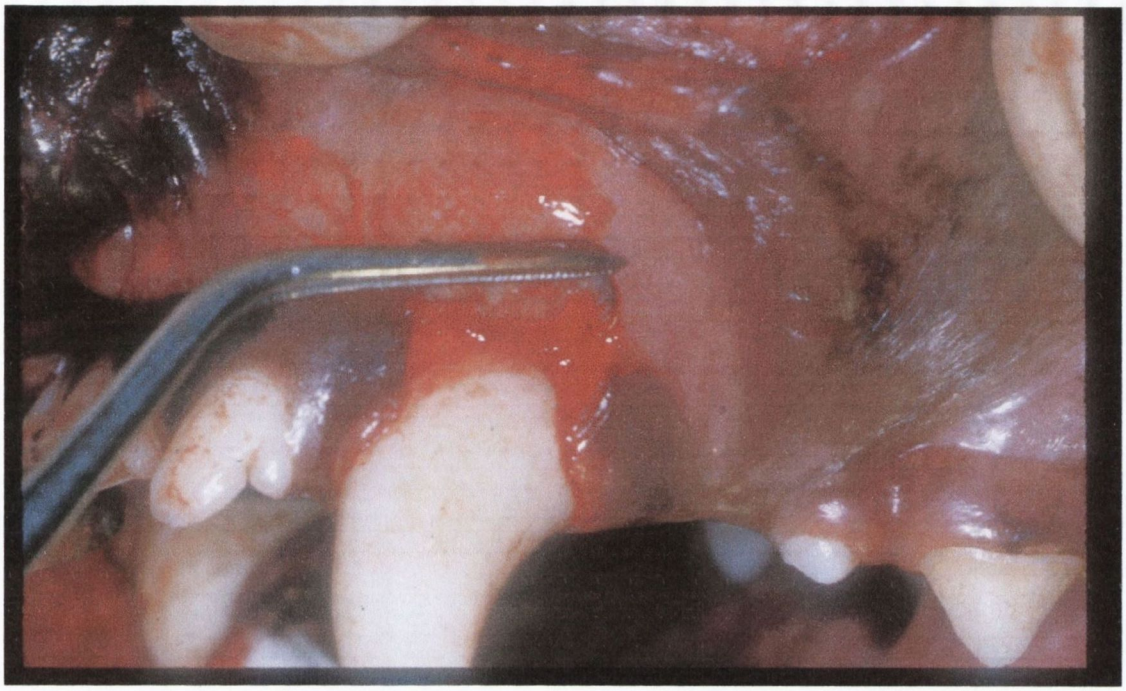


Figure 3.7.1 Surgical wound pocket creation model on canine tooth.

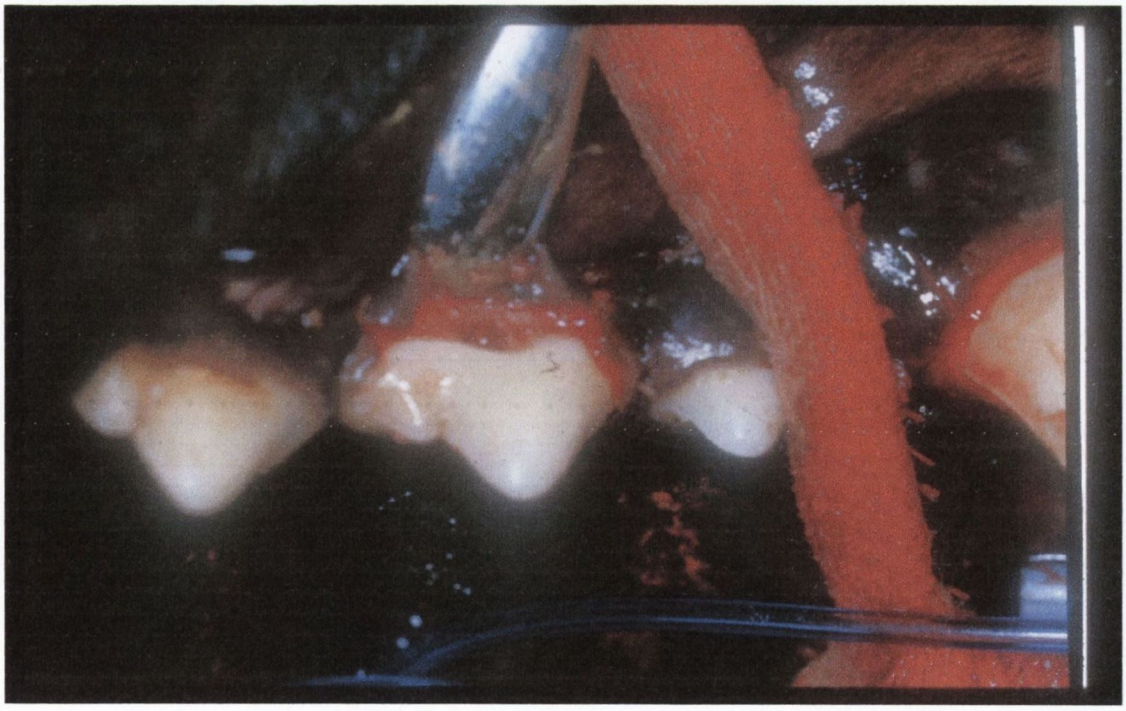


Figure 3.7.2 Furcation defects surgically created on the premolar tooth.

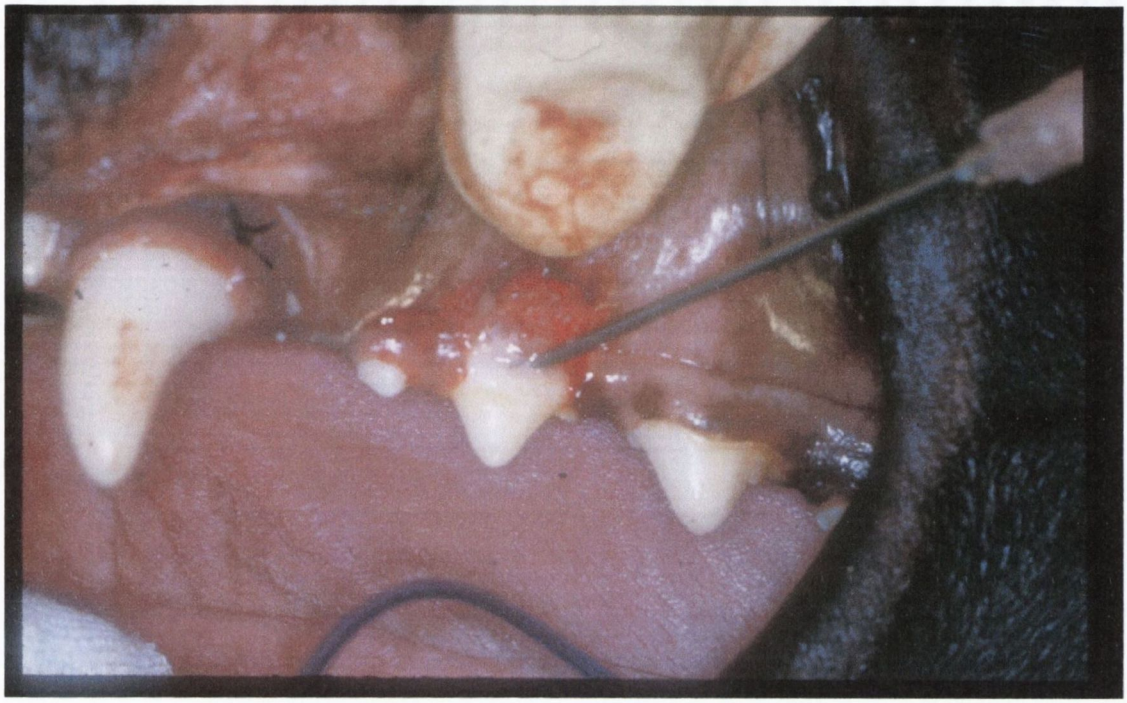


Figure 3.7.3 Injection of the final formulation into the bottom of the pocket.

3.7.4 Microbiological testing

24 x 5 ml Regina vials were autoclaved and filled with 2 ml sterile half-strength Ringers solution using sterile pipette tips and a 1 ml finpipette (Figure 3.7.4). The pockets were sampled using sterile orthodontic points, which were inserted into the bottom of each periodontal pocket until resistance was met and kept in place for 30 s (Figure 3.7.5). They were placed in the vials immediately after sampling. These were shaken for 15 s and a 1 in 10 dilution was performed using half-strength Ringers solution. 200 µl quantities were plated on agar plates. The growth medium contained brain heart infusion broth (0.35%) gelled with 1% agar. Plates were stored under aerobic and anaerobic conditions with total colony forming units (CFU's) being counted after 24 hr incubation at 37°C for aerobic plates and 48 hr at 37°C for anaerobic plates.

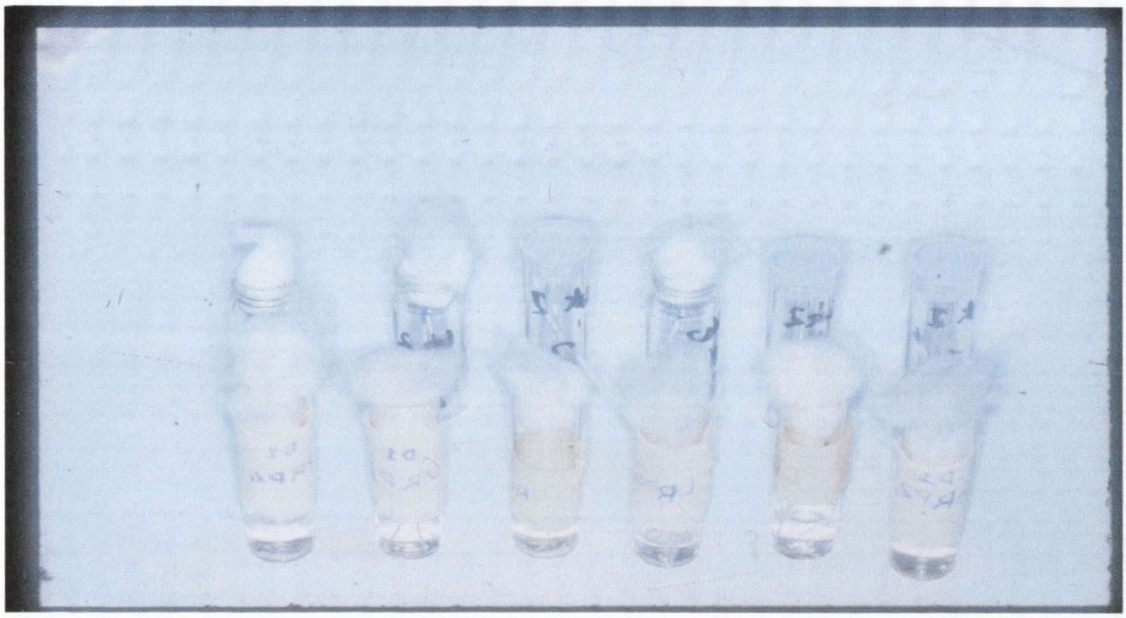


Figure 3.7.4 Endodontic points in half-strength Ringers solution after microbiological sampling.

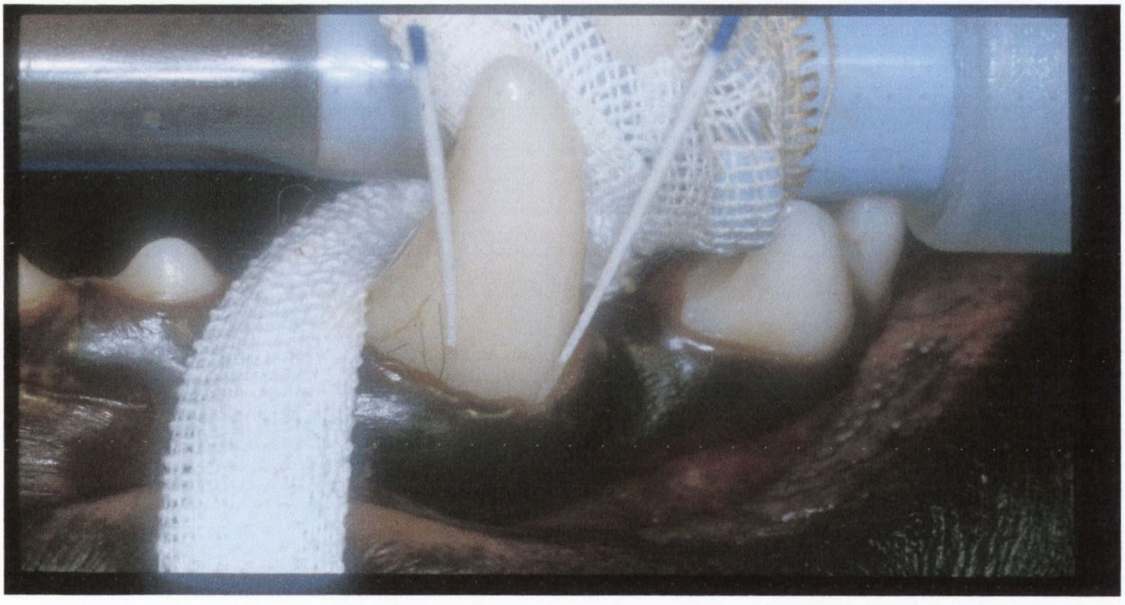


Figure 3.7.5 Sampling of periodontal pockets for microbiological and tetracycline residue testing.

3.7.5 Tetracycline residue testing

24 x 5 ml Regina vials were weighed on a Mettler Toledo MT5 microbalance. 2 sterile orthodontic points were placed in each of the vials, which were then reweighed (Figure 3.7.6). Two endodontic paper points were placed into each periodontal pocket and kept in place for 30 s. The two paper points were returned to the vial. After the pockets were sampled, the vials were once again weighed to determine the weight of tetracycline residue obtained on the orthodontic points (Appendix 8). The samples were frozen at – 20°C until analyses was performed on them.

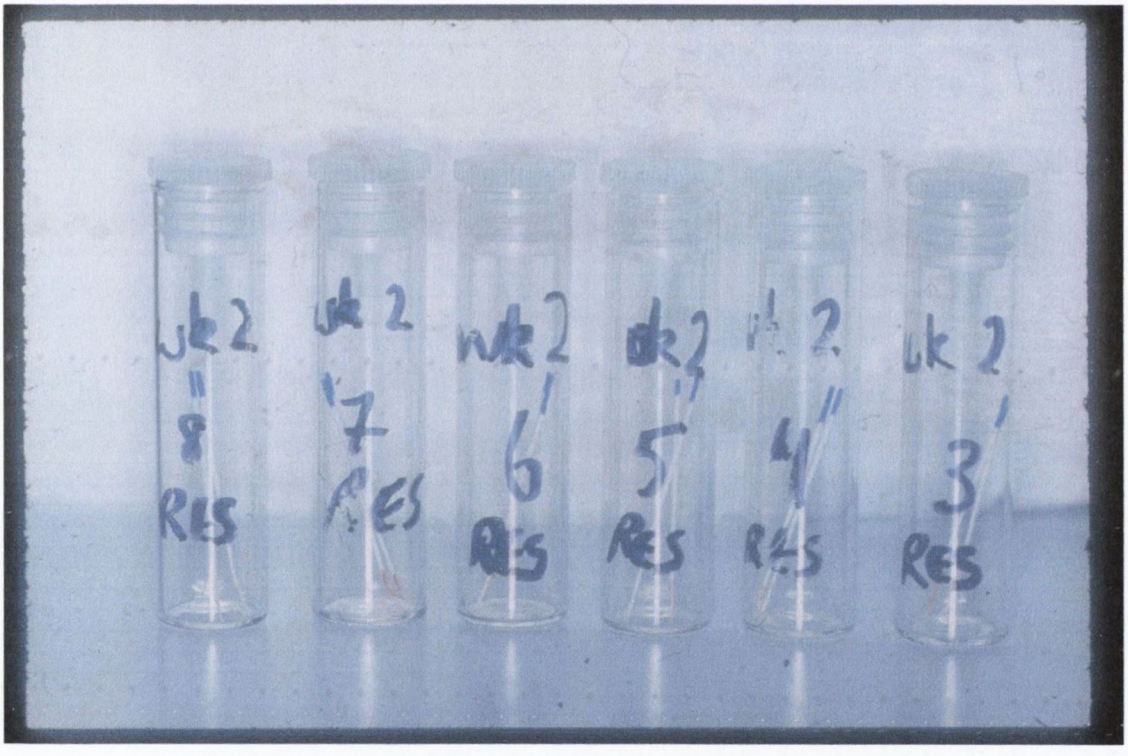


Figure 3.7.6 Vials showing 2 sterile endodontic points used for sampling pockets for tetracycline residue content.

3.7.6 Probing depths

Probing pocket depths of test and control pockets were recorded at all sampling time points using a conventional periodontal probe. Recordings were made at one site for canine and non-molar teeth. Midbuccal sites were measured by placing the probe at these locations, and directing it longitudinally along the root surface (Figure 3.7.7).

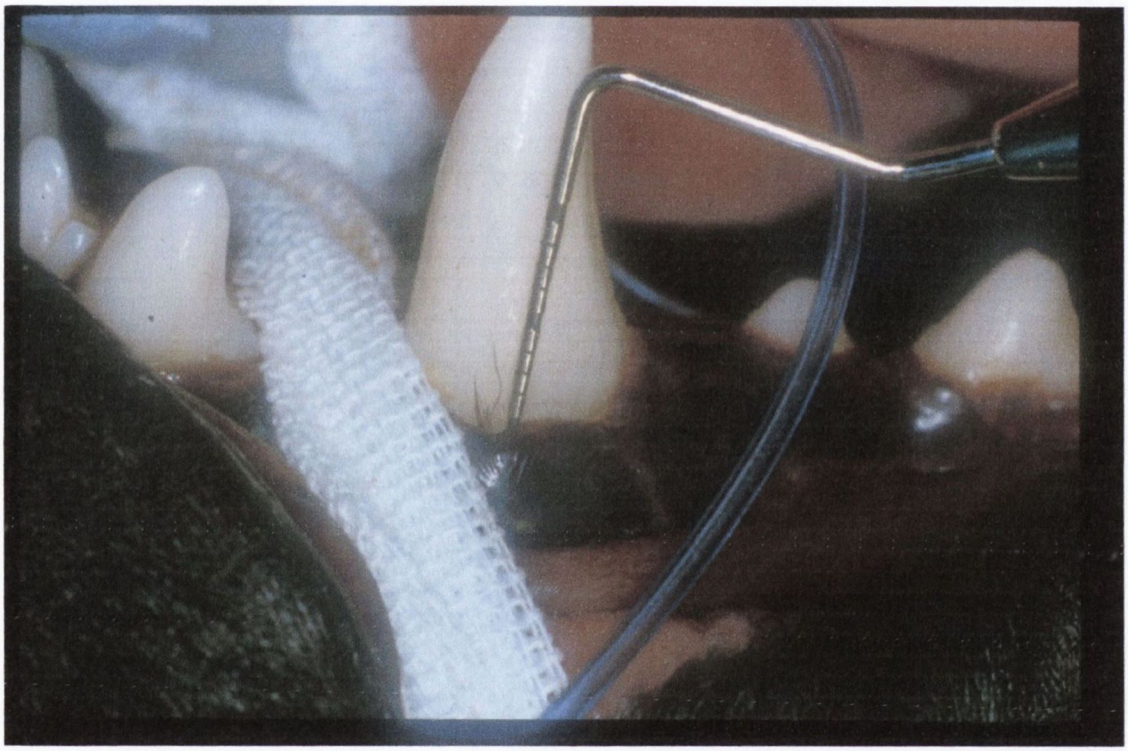


Figure 3.7.7 Measurement of probing depth.

3.8 Formulation of a product for the treatment of xerostomia

3.8.1 Introduction

The aim of these experiments was to develop a product to help alleviate the symptoms associated with xerostomia. Xerostomia occurs when there is a partial or complete failure of saliva production in the oral cavity. The product being developed is essentially a salivary replacement or oral lubricant. It is important that the product tries to imitate as many of the functions of saliva as possible and the formulation developed sought to reflect this need. Also the product must offer permanence, which requires the presence of a bioadhesive polymer. The polymer chosen for use in these formulations was *Carbopol 974P*™. It was chosen due to its good bioadhesive properties (Smart *et al.*, 1984) and good safety profile (BF Goodrich safety data sheets).

3.8.2 Method for preparation of formulations

The formulations (Table 3.8.1 - Table 3.8.3) were all prepared as outlined in the following method:

The relevant quantities of sodium fluoride, calcium chloride, potassium chloride, sodium chloride, magnesium chloride, monobasic potassium phosphate and zinc sulphate were placed in a pre-weighed beaker. 20 ml approx. of distilled water was added followed by the required quantities of xylitol, sorbitol and flavoring agent. The contents were stirred continuously, using a magnetic stirrer, until fully dissolved. The required quantity of *Carbopol 974P*™ was added slowly, with continuous stirring over 60 min and left overnight at room temperature to allow complete hydration of the *Carbopol 974P*™. In a separate beaker, sodium lauryl sulphate and triclosan were placed in 10 ml approx. of distilled water, 1.0 ml of triethanolamine was added and the preparation was sonicated until the triclosan was fully solubilized. The solubilized triclosan solution was added to the hydrated polymer system, which was partially neutralized by the triethanolamine present in the triclosan solution. The pH was further adjusted to pH 6.75 ± 0.05 using

triethanolamine. The formulation was adjusted to its final weight using distilled water and the pH was checked again. If oil was present in the formulation, distilled water was added to give the weight required before the addition of the oil. The oil was added and emulsification was carried out using an Ultra-Turrax T25 shear rate mixer at 7,500 rpm for a period of 4-5 min. The pH of the emulsion was again checked to ensure it was within the range 6.75 ± 0.05 and adjusted as required using triethanolamine (Table 3.8.1 - Table 3.8.3). The product was centrifuged at 2,000 rpm for 10 min to remove any air bubbles trapped in the emulsion. The recovered emulsion was placed in a glass screw top jar and stored at 20°C prior to use

Carbopol 974P ™ gel alone was prepared by adding the required quantity of polymer to a pre-weighed beaker containing approximately 90 ml of distilled water. This was left to stir for 60 min and was allowed to hydrate overnight. It was adjusted then to pH 6.75 ± 0.05 using triethanolamine and made to final weight with distilled water. The pH was checked again.

Table 3.8.1 Formulation of various products prepared for examination as saliva substitutes.

Formulation	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
Sodium fluoride (mg)	0.42	0.42	0.42	////////////	////////////	////////////	////////////	////////////	////////////	////////////
Calcium chloride (g)	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166
Potassium chloride (g)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
Sodium chloride (g)	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844
Magnesium chloride (g)	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058
Potassium phosphate (g)	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366
Xylitol (g)	3.0	5.0	5.0	////////////	////////////	////////////	8.0	8.0	10.0	15.0
Sorbitol (g)	////////////	////////////	////////////	3.0	5.0	5.0	5.0	5.0	10.0	5.0
Zinc sulphate (g)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Triclosan (g)	0.15	0.3	0.15	0.15	0.1	0.15	0.1	0.1	0.1	0.1
Na lauryl sulphate (g)	1.0	2.0	1.0	0.5	0.5	0.75	1.0	1.0	1.0	1.0
Lemon flavour (g)	0.25	0.25	0.25	0.25	0.2	0.2	0.25	0.25	0.4	0.3
Carbopol 974P ™(g)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Distilled water	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g

Table 3.8.2 Formulation of various products prepared for examination as saliva substitutes.

Formulation	***11***	***12***	***13***	***14***	***15***	***16***	***17***	***18***	***19***	***20***
Sodium fluoride (mg)	0.42	0.42	0.42	0.42	0.42	////////////	////////////	0.42	0.42	0.42
Calcium chloride (g)	0.0166	0.0166	0.0166	0.0166	0.0166	////////////	////////////	0.0166	0.0166	0.0166
Potassium chloride (g)	0.12	0.12	0.12	0.12	0.12	////////////	////////////	0.12	0.12	0.12
Sodium chloride (g)	0.0844	0.0844	0.0844	0.0844	0.0844	////////////	////////////	0.0844	0.0844	0.0844
Magnesium chloride (g)	0.0058	0.0058	0.0058	0.0058	0.0058	////////////	////////////	0.0058	0.0058	0.0058
Potassium phosphate (g)	0.0366	0.0366	0.0366	0.0366	0.0366	////////////	////////////	0.0366	0.0366	0.0366
Xylitol (g)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	////////////	////////////	15.0
Sorbitol (g)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	////////////	////////////	5.0
Zinc sulphate (g)	0.05	0.05	0.05	0.05	0.05	////////////	////////////	0.05	0.05	0.05
Triclosan (g)	0.1	0.1	0.1	////////////	////////////	0.1	0.1	0.1	0.1	0.1
Na lauryl sulphate (g)	1.0	1.0	1.0	////////////	////////////	1.0	1.0	1.0	1.0	1.0
Lemon flavour (g)	0.3	0.3	0.4	0.4	0.400	0.4	0.4	0.4	0.4	0.5
Olive oil (g)	////////////	////////////	////////////	////////////	////////////	////////////	////////////	////////////	////////////	5.0
Carbopol 974P ™(g)	2.0	1.0	0.5	1.0	0.5	1.0	0.5	1.0	0.5	1.0
Distilled water	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g

Table 3.8.3 Formulation of various products prepared for examination as saliva substitutes.

Formulation	***21***	***22***	***23***	***24***	***25***	***26***	***27***	***28***	***29***	***30***
Sodium fluoride (mg)	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42
Calcium chloride (g)	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166	0.0166
Potassium chloride (g)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
Sodium chloride (g)	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844	0.0844
Magnesium chloride (g)	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058	0.0058
Potassium phosphate (g)	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366	0.0366
Xylitol (g)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Sorbitol (g)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Zinc sulphate (g)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Triclosan (g)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
N lauryl sulphate (g)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lemon flavour(g)	0.5	0.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Olive oil (g)	10.0	20.0	5.0	10.0	////////////	////////////	////////////	////////////	////////////	////////////
Sunflower oil (g)	////////////	////////////	////////////	////////////	////////////	////////////	5.0	10.0	15.0	20.0
Carbopol 974P ™(g)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Distilled water	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g	to 100 g

3.9 *In vitro* testing for bioadhesive strength

3.9.1 Introduction

Bioadhesive strength of the lead xerostomic formulations was determined *in vitro* using a texture analyzer XT.RA (Stable Micro Systems). The apparatus consists of an upper metal probe (surface area 5.12 cm^2) to which the mucosa or mucus is attached. The sample is placed in a secure position on the lower platform. The probe is lowered at a fixed speed until it comes in contact with the sample with a defined force, where it is held for a defined period of time. At the end of this time period, the probe retracts at a defined speed breaking the bioadhesive bond. This procedure is carried out under the adhesive mode. A profile of force *vs.* time is obtained from which the maximum force of detachment F_{max} is obtained in mN. The work of adhesion is determined from the area under the curve (N.mm).

3.9.2 Models for bioadhesion testing

Three different models for bioadhesion were examined, dried porcine gastric mucin (DPGM), fresh porcine gastric mucus (FPGM) and fresh porcine oral mucosa (FPOM). Dried porcine gastric mucin (20% w/w) was prepared by adding the required quantity of dried powder to distilled water and allowing it to hydrate overnight at 4°C. The fresh porcine gastric mucus was extracted from freshly slaughtered porcine stomach, using the method as described in Section 3.9.3. Fresh porcine oral mucosa was obtained from freshly slaughtered pigs (within one hour of slaughter) from Coyle Bros., Garden Lane, Dublin 1. The top layer of tissue was excised from the lower connective tissue layers and the tissue cut into suitable sample sizes. The tissue was either used immediately or frozen at -20°C with greaseproof paper separating individual samples of tissue. The tissue was defrosted at 4°C as required and used immediately.

3.9.3 Preparation of homogenized fresh porcine gastric mucus (FPGM)

The entire procedure outlined in this section was performed in a fume hood. The interior of the fume hood was washed with ethanol before and after the procedure as were all instruments used in the process. Protective clothing, safety glasses and double gloving were used as further precautionary measures. All samples were properly disposed of when finished with. Batches of crude mucus were obtained by scraping porcine stomachs received fresh from slaughter (Coyle Bros., Pork Wholesalers) and purified using a method modified from Mortazavi *et al.*, 1993. The crude mucus was collected in a glass jar to which an equal quantity of an isotonic solution containing phenylmethylsulphonylfluoride (PMSF) (0.0175% w/v), sodium azide (0.02% w/v), ethylenediaminetetracetic acid (EDTA) (0.186% w/v) and sodium chloride (0.9% w/v) was added. This was then homogenized by blending for 4 min. The resulting mixture was placed in 10 ml glass centrifuge tubes and centrifuged at 3,000 *g* for 1 hr 30 min at 4°C using a temperature-controlled centrifuge (Sorvall, UK). Dialysis membrane (Medicell, cut off weight 12,000-14,000 Da) was prepared for use by heating in a large volume of 2% w/v sodium bicarbonate and 1 mM EDTA at 80°C for 30 min. The tubing was then thoroughly rinsed with distilled water. Where the membrane was not used immediately, it was stored in a 0.1% w/v sodium azide solution at 4°C and rinsed thoroughly with distilled water prior to use. The gel layers were removed from each centrifuge tube, pooled and exhaustively dialyzed for 24 hr at 4°C in a large volume of distilled water, with two water changes over twenty four hours and finally homogenized by blending. If the mucus was not to be used immediately, it was frozen at -20°C until required and it was then defrosted overnight at 4°C.

3.9.4 Bioadhesive polymers for use in bioadhesive sodium fluoride tablet

A number of different systems were considered for the lower bioadhesive layer of the sodium fluoride tablet (Table 3.9.1). Discs were prepared by mixing the two components in the desired proportions on a weight per weight basis using a mortar and

pestle. 300 mg of the resulting mix was compressed into discs of diameter 13 mm using a Perkin-Elmer IR press with a flat–faced 13 mm punch and die set, at a pressure of 2 ton for 1 min.

Table 3.9.1 Bioadhesive polymers considered for use in sodium fluoride tablet.

Sample no.	***System***
1	100% w/w drum dried waxy maize starch (DDWMS)
2	95% w/w DDWMS, 5% w/w *Carbopol 974P*™
3	90% w/w DDWMS, 10% w/w *Carbopol 974P*™
4	95% w/w DDWMS, 5% w/w chitosan
5	90% w/w DDWMS, 10% w/w chitosan
6	100% w/w chitosan

3.9.5 Testing of samples for bioadhesive strength

The systems tested for bioadhesion are outlined in Table 3.9.2.

When using FPGM or DPGM, the probe was covered with a thin layer of either FPGM or DPGM, which was then dried using warm air (approx. 40°C). For porcine oral mucosa a thin layer of the tissue was attached to the probe using an adhesive backing layer. Systems 1 and 2 (Table 3.9.2) were placed in a 60 ml glass jar to a depth of 20 mm. The container was anchored to the platform during testing using double-sided adhesive tape. When testing the bioadhesiveness of the discs, 15 µl of phosphate buffer pH 6.8 was pipetted onto the surface of the discs prior to testing. The probe was brought in contact with the test samples with a force of 0.1 N and held there for a specified period of time, then withdrawn at a speed of 0.1 mm/s. Five replicates were performed for each sample.

Table 3.9.2 Systems tested for bioadhesion and the models used for their testing.

Systems	***Sample no.***	***Bioadhesion model used***		
		DPGM[1]	FPGM[1]	FPOM[1]
1	*Carbopol 974P*™ 1% and 0.5%	Yes	Yes	Yes
2	Prod. 12 (Table 3.6.2), 23 and 27 (Table 3.6.3)	Yes	Yes	Yes
3	Sample no. 16 and 20 (Table 3.1.1)			Yes
4	Samples 1 – 6 (Table 3.7.1)			Yes

3.10 Measurement of surface pH of bioadhesive layer

The surface pH of the bioadhesive tablets was evaluated in order to determine the possibility of mucosal irritation *in vivo* (Bottenberg *et al.*, 1991). Discs were made as outlined in Section 3.9.4. The discs were allowed to swell for 2 hr in 1 ml of distilled water (pH 6.4 ± 0.05) in glass vials. A combined glass pH electrode (AGB 2000 pH meter) was brought in contact with the disc and the pH measured after allowing 5 min for equilibration.

3.11 Friction testing of xerostomic products

3.11.1 Introduction

Friction testing was performed in the Department of Mechanical Engineering, Trinity College, Dublin on a piece of apparatus that had been constructed in-house (Figure 3.11.1). It consisted of a lower fixed plate onto which the test substrate and test substance could be placed and an upper movable arm to which different geometries

could be attached. The upper arm moved along the lower plate at varying speeds and differing distance creating friction between the two surfaces that could be measured and analyzed using in-house developed computer software.

3.11.2 Method of testing

A piece of perspex® 10 cm in length was covered with a soft piece of leather using cyanoacrylate adhesive. The leather was used to simulate the oral mucosa (Blanco-Fuente *et al.*, 1996b). The upper probe was also covered with the same leather. The sample was loaded onto the 10 cm plate and spread evenly over it. The instrument was put into run mode and the probe moved at a set speed up and down the fixed 10 cm plate for a defined period of time, measured in seconds. All samples were examined at the same speed and distance of travel. The friction was measured in terms of the friction coefficient, μ. This method was performed on *Carbopol 974P™* 1% w/w, Product 12 (Table 3.8.2) and Product 23 (Table 3.8.3).

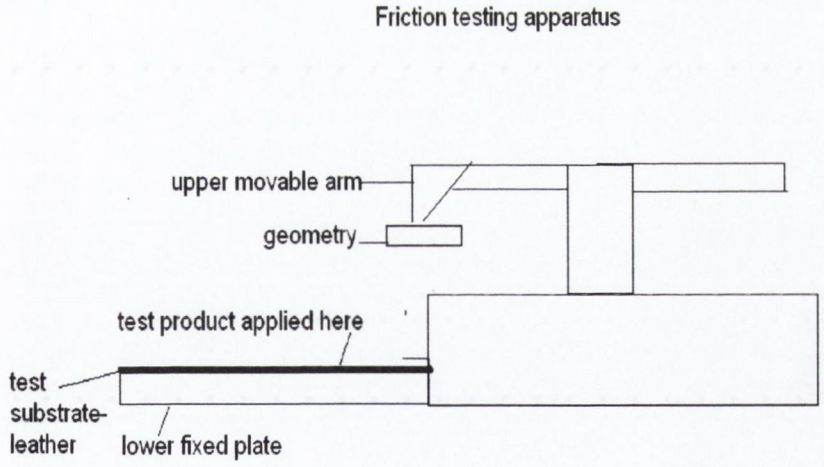


Figure 3.11.1 Diagrammatic representation of friction testing apparatus.

3.12 Rheometry

3.12.1 Introduction

Rheological properties of various systems were evaluated using a Carri-Med rheometer CSL^2500 (TA Instruments, UK), which is a controlled stress/controlled rate rheometer capable of handling numerous types of samples and a range of geometry types and sizes. This rheometer has an air bearing, i.e. it uses air as its lubricating medium, which permits virtually friction free application of torque, allowing it to move under the smallest of forces. The pressurized air is passed through a coarse filter desiccant dryer system and a double filter regulator assembly, to remove particulate matter and ensure a sufficiently pure dry air supply. The air is delivered at a stable pressure of 37.5 psi. A peltier plate is temperature controlled via a constant water supply, that allows rapid and accurate heating and cooling of samples. An automatic gap was determined by the instrument that was 42 μm. The automatic gap setting mechanism was used in order to minimize manual operating errors and reduce operating time.

3.12.2 Method

Two types of rheometry were performed, oscillatory and continuous shear.
The oscillatory technique is theoretically a non-destructive test, which measures the viscous and elastic behavior of a sample simultaneously. When performing oscillatory frequency sweeps, the linear viscoelastic region of the system must be first determined by torque sweep. Oscillatory measurements were performed on the systems outlined in Table 3.12.1 and Table 3.12.2. Continuous shear measurements to determine apparent viscosity were also performed on a number of different systems outlined in Table 3.12.3.

All experiments used cone and plate test geometry (angle 4°), which is ideally suited for single-phase homogeneous samples or samples with sub-micron particles. Sample size is determined by the geometry used, as it must spread to the edges of the geometry in use.

Proper loading of the sample is not only based on sample size but also on correct positioning of the sample on the peltier plate. An equilibration time of 5 min was allowed after loading the sample on the peltier plate before each experimental run, in order to allow internal stresses caused by sample loading to dissipate and to ensure a uniform temperature throughout the sample. Each experiment was run in triplicate, with a fresh sample loaded for each run.

Table 3.12.1 *Poloxamer 407* systems on which oscillatory rheometry was performed.

Sample number (Table 3.1.1)	***Frequency (Hz)***	***Temperature (°C)***	***Oscillatory torque (μN.m)***
1	1	20	100-2000
1	0.1	15-45	1350
1	1	15-45	1350
1	5	15-45	1350
2-7, 9	1	15-45	1350
8	1	15-35	1350
10-21	1	15-40	1350

Table 3.12.2 *Carbopol 974P*™ systems and commercial products on which oscillatory rheometry was performed.

System	***Frequency range (Hz)***	***Temperature (°C)***	***Oscillatory torque (μN.m)***
Carbopol 974P ™ 1% Neutralized *Carbopol 974P* ™1% Unneutralized *Carbopol 974P* ™ 0.5%	1-10	20	1500
Carbopol 974P ™ 1% Unneutralized *Carbopol 974P* ™ 1%	1-40	20	1500
Carbopol 974P ™1%, *Carbopol 974P* ™ 0.5%, *Luborant*™, *Oralbalance*™	1	37	500-2000
Carbopol 974P ™ 1%, *Carbopol 974P* ™ 0.5% Products 12, 14, 16, 20, 21, 22, 25, 26, 27 and 28 (Tables 3.6.2 and 3.6.3)	0.01-10	37	1500
Luborant ™	1-10	37	1500
Oralbalance ™ *Bioxtra*™	0.01-10	37	1250
Products. 13, 15 and 17 (Tables 3.6.2 and 3.6.3)	0.5-10	37	1500

Table 3.12.3 Systems that had continuous shear determinations performed.

Sample no.	***System***
1	Unneutralized *Carbopol 974P ™* 0.5%
2	Neutralized *Carbopol 974P ™* 0.5%
3	Unneutralized *Carbopol 974P ™* 1%
4	Neutralized *Carbopol 974P ™* 1%
5	*Luborant ™*
6	*Oralbalance ™*
7	*Poloxamer 407* 20% w/w, octyl CA 1% w/w, PEG 20,000 0.5% w/w, halloysite double loaded with tetracycline base and coated with chitosan 200 mg/ml.
8	*Poloxamer 407* 20% w/w, octyl CA 1% w/w, PEG 20,000 0.5%
9	*Poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w

3.12.3 Rheological synergism with fresh porcine gastric mucus (FPGM)

2 g samples of FPGM were mixed with an equal quantity of the relevant system (Table 3.12.4) and the pH was adjusted to 6.75 using 10% w/v triethanolamine. The final weight of the sample was adjusted to 4.5 g using distilled water. Further mixtures containing 2 g of each system were adjusted to pH 6.75 and made up to 4.5 g using distilled water. A sample containing mucus 2 g and distilled water 2.5 g was also prepared, to act as a control. Oscillatory rheometry (triplicate determinations) was carried out on all of the samples listed in Table 3.12.4, using a frequency sweep of 0.1-10 Hz at 37°C, with an oscillatory torque of 1350 μNm, which was determined to be within the linear viscoelastic range.

Table 3.12.4 *Carbopol 974P™* based systems (Table 3.6.2 and 3.6.3) examined for rheological synergism.

System	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Porcine gastric mucus	2.0 g	2.0 g	2.0 g	2.0 g			
Product 12	2.0 g				2.0 g		
Product 23		2.0 g				2.0 g	
Carbopol 974P™ 1%			2.0 g				2.0 g
pH (adjusted to)	6.75	6.75	6.75		6.75	6.75	6.75
Distilled water to	4.5 g	4.5 g	4.5 g	4.5 g	4.5 g	4.5 g	4.5 g

3.13 Statistical analysis

3.13.1 Simple comparative experiments

Simple comparative t-tests were applied to situations where two independent samples were involved. All tests were conducted at 0.05 level of significance. The standard error was calculated using a pooled estimate of the sample variance. The hypothesis was tested with a t-ratio complying with a students t-distribution having n - 1 degrees of freedom, where n is equal to the sample number for each sample group. The t-ratio for two sample means is given by

$$t = \frac{\bar{Y}_2 - \bar{Y}_1}{S_D} \qquad \text{Equation 3.13.1}$$

where t is the ratio

Y_2 is the mean of sample 2

Y_1 is the mean of sample 1

S_D is the estimated standard error of the mean difference

3.13.2 Analysis of variance (ANOVA)

Where the t-test is used to perform comparisons between more than two sample means it is known as analysis of variance (ANOVA). ANOVA is used for an overall test for more than two sample means for quantitative data. The ANOVA tables used in the results sections are divided into the following columns:

Source is the source variation

df is the degrees of freedom

SS is the sequential sum of squares

MS is the adjusted mean squares

F is the *F*-statistic

P is the *p*-value which is the probability associated with the *F* statistic

The *F*-statistic in an ANOVA table are based on the adjusted sum of squares, which are equivalent to the adjusted mean squares multiplied by the respective degrees of freedom. The *F*-statistic is calculated by dividing the mean square value for the respective term by the mean square value for residual error. A high *F*-statistic associated with a low *p*-value (<0.05) would be considered significant.

3.13.3 Factorial experiments

Full factorial experiments were designed by the following nomenclature:

$$N = L^k$$

where K is the number of variables

L is the number of variable levels

N is the number of experimental runs

The experiment allows for the determination of the main effects of each variable and also interactions between factors.

3.13.4 Modelling of release profiles

The experimental release data from the drug loaded microparticles in chapter 7 was fitted to various theoretical models and equations using MINSQ, a nonlinear curve fitting and model development program from MicroMath™ Scientific Software. The ability of each equation to describe the experimental data was assessed using both the Coefficient of Determination (CoDet) and the Model Selection Criterion (MSC). The CoDet is a measure of the fraction of the total variance accounted for by the model and is taken as a measure of goodness of fit. The MSC represents the 'information content' of a given set of parameter estimates by relating the CoDet to the number of parameters that were required to obtain the fit. When comparing two models with different numbers of parameters, this criterion places a burden on the model with more parameters to have a better CoDet. The most appropriate model is that with the highest MSC.

Chapter 4

Formulation of a local drug delivery system for the treatment of periodontitis

4.1 Anti-microbial treatment of periodontitis

Periodontitis is a localized inflammation of the periodontal pocket caused by bacterial infection, which can result in tooth loss. The current microbiological treatment of periodontitis is through either the use of systemic antibiotics or a localized delivery system incorporating an antibiotic. The use of systemic antibiotics in the treatment of periodontitis raises a number of issues. Large doses must be taken in order to achieve sufficient concentrations in the gingival crevicular fluid of the periodontal pockets; this brings with it the associated side effects of antibiotics and problems regarding antibiotic resistance (Van Winkelhoff *et al.*, 2000). There are also concerns regarding compliance, with problems increasing with frequent dosing or prolonged treatment. As a result of these issues, treatment of periodontitis with localized drug delivery systems is becoming more prevalent. It is considered that there are a number of properties that formulations for the localized treatment of periodontitis should possess (Jones *et al.*, 2000):

- controlled drug release over a prolonged period
- acceptable mechanical properties to ensure ease of administration
- mucoadhesive properties to ensure retention of the formulation

However products currently available do not necessarily offer all of these properties. Application to the pocket can be intricate and time consuming, requiring considerable expertise, maximum release is over 10 days only and retention in the periodontal pocket, in certain cases, can be a problem. It was felt that a more optimum formulation could be developed. The novel excipient, halloysite, was used to provide a controlled release of tetracycline base, while a thermoresponsive polymer delivery system was developed to improve ease of application and pocket retention. This product would provide a syringeable formulation easily applied, with a suitable drug delivery profile, which is capable of being retained in the pocket for a prolonged period of time.

4.2 Morphology of halloysite samples

Halloysite is an aluminosilicate material, chemically similar to kaolin. However morphologically, it has a hollow tubular structure, unlike kaolin, which has a stacked-plate structure. This hollow cylindrical structure can be loaded with drug, which is then gradually released, offering a sustained release mechanism. The *in vitro* release characteristics of a number of drugs from halloysite, including tetracycline HCl, have been previously examined by Price *et al.*, (2001) and shown to have sustained release properties.

Figure 4.2.1, Figure 4.2.2 and Figure 4.2.3 are scanning electron micrographs (SEM's) at different magnifications, of a typical halloysite sample. Figure 4.2.1 (X100) shows the halloysite to be a powder, which forms aggregates of varying sizes. Figure 4.2.2 (X20, 000) and Figure 4.2.3 (X100, 000) show the tubular nature of halloysite with small particle size, and a wide degree of variation in the length of tubules present. Diameters have been shown to be typically of 50 nm to 0.3 μm and lengths between 1 and 20 μm (Price and Gaber, 1997). The outer surface of halloysite normally carries a negative charge, whereas the ends of the tubule carry a positive charge. Drug loading occurs through two mechanisms, firstly and most importantly the entrapment of the drug in the hollow tubules and secondly, adsorption of cationic drug onto the surface of the tubules. Release of the active agent is probably first from the exterior surfaces of the halloysite by desorption, if binding is reversible, followed by a more prolonged phase of release, mainly by diffusion from the ends of the microtubules (Price *et al*, 2001). The halloysite as can be seen from the SEM (Figure 4.2.2) has a wide range of tubular sizes, with much of the tubular material being of small particle size. This smaller particulate material is less useful for drug loading as it is fragmented and not able to take up significant amount of drug, due to its lack of tubular space. It would be preferable if halloysite samples containing larger proportions of long tubules could be obtained, as it would give greater drug loading and also more uniform and consistent drug loading.

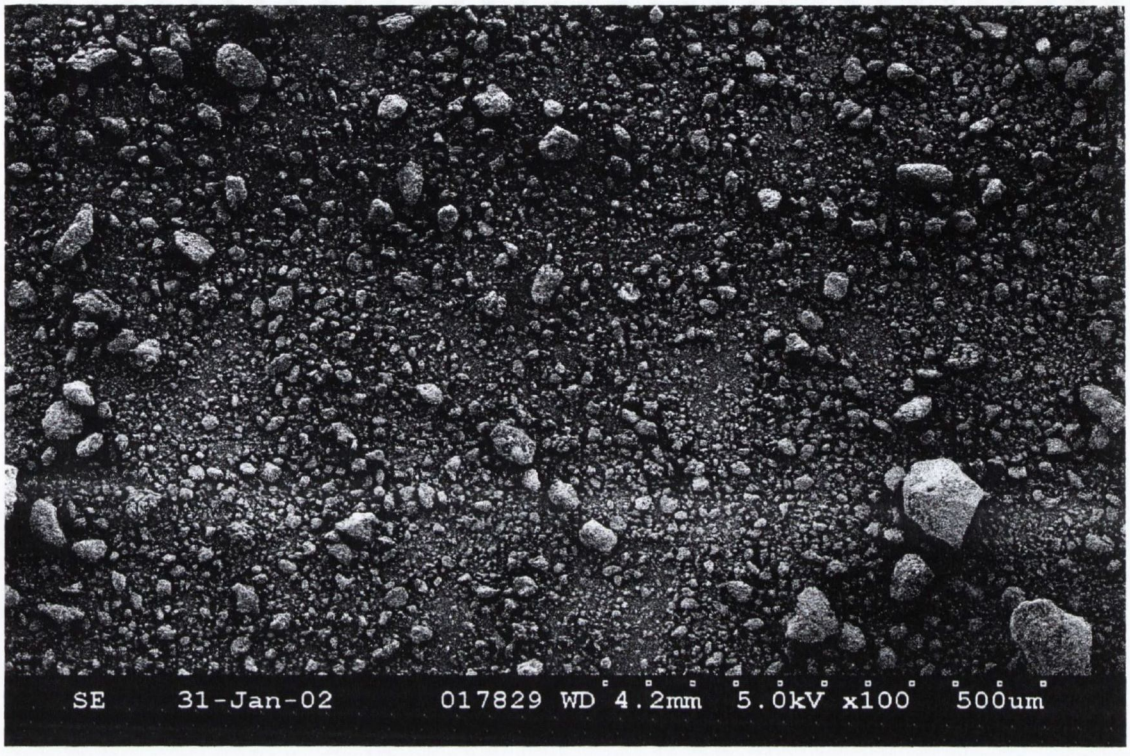


Figure 4.2.1 SEM of halloysite (X100).

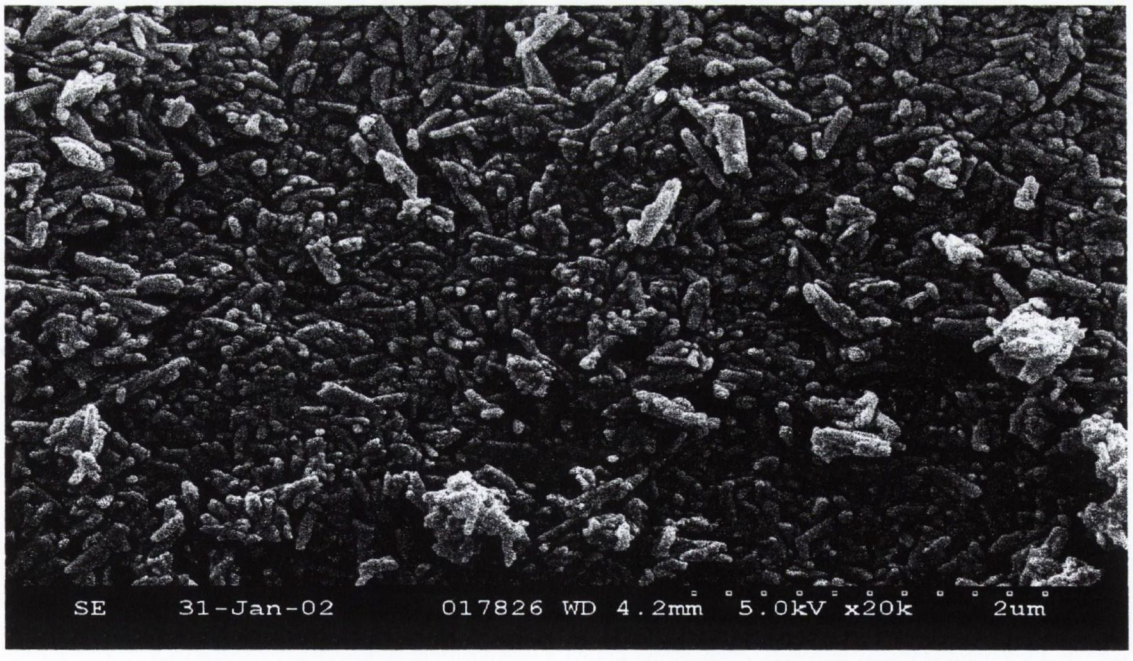


Figure 4.2.2 SEM of halloysite (X20,000).

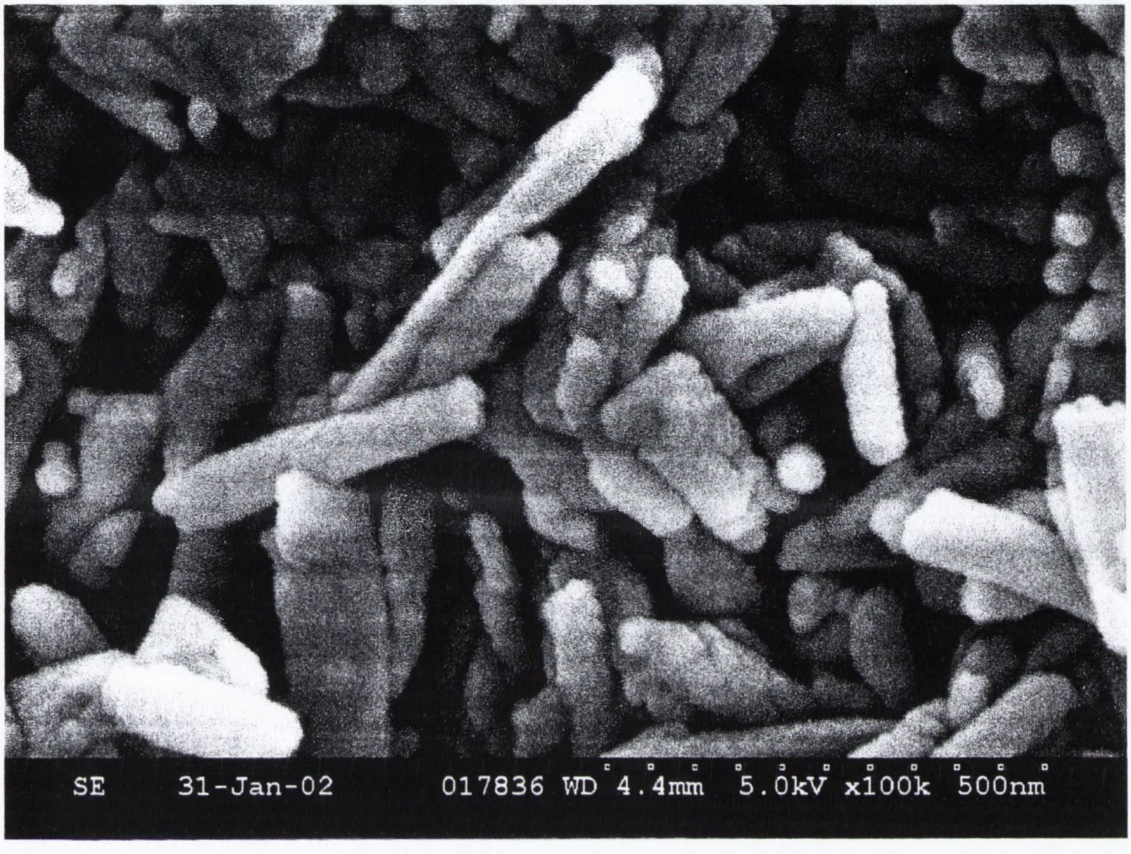


Figure 4.2.3 SEM of halloysite (X100,000).

4.3 Chitosan

Coating the halloysite using cationic polymers that bind onto the negative charge present on the tubules i.e. charge neutralization, can further enhance the retardation of drug release. The cationic polymer chosen for use was chitosan. Chitosan has been previously shown to enhance the retardation of drug release from halloysite (Levis, 2000). Chitosan is the N-deacetylated product of chitin, one of the most abundant polymers found in nature after cellulose, being a structural component of shellfish, insects and the cell walls of bacteria and mushrooms. It is a copolymer of N-acetylglucosamine and glucosamine units, with glucosamine units usually dominant. Chitosan is a cationic polyelectrolyte and has been shown to be both biocompatible and biodegradable (Hirano *et al.*, 1988, Orienti *et al.*, 1996). Due to these properties it has

been examined for a variety of biomedical applications. Schnürch, (2000) reviewed the use of chitosan as an excipient for use in peroral peptide delivery systems. It has been shown that chitosan has a permeation enhancing effect for peptide drugs. The polymer can also have enzyme inhibitors embedded on the polymer offering a protective effect from intestinal peptidases. Due to these features chitosan can offer a strongly improved bioavailability of many perorally given peptide drugs such as insulin and calcitonin.

4.4 Tetracycline and the encapsulation efficiency it achieves in halloysite

4.4.1 Tetracycline

Tetracycline was the model drug chosen for the formulation of a product for the localized treatment of periodontitis. It was chosen for a number of reasons. It is already widely used in the treatment of periodontitis both systemically and locally, and has been shown to be effective against many of the common periodontopathic bacteria, in particular against *Prevotella intermedia* and *Porphyromonas gingivalis* (Miyake *et al.*, 1995, Baker *et al.*, 1985, Gordon *et al.*, 1981) and has also been shown to inhibit tissue collagenase activity (Golub *et al.*, 1984). Systemically it has been used for the treatment of periodontitis (American Academy of Periodontology, 1996) at doses of 500 or 1000 mg daily.

Tetracycline is also currently used in a commercially available product for the localized treatment of periodontitis, known as '*Actisite*™'. *Actisite*™ has had numerous large scale clinical studies performed (Lowenguth *et al.*, 1995, Goodson *et al.*, 1991b), further verifying the positive impact that a tetracycline delivery system has on the clinical parameters associated with periodontitis. Tetracycline solutions have been shown to have good binding capacity to tissue in the periodontal pocket, resulting in the antibiotic showing a prolonged half-life of 4.4 hr and 12.2 hr (1% and 10% solutions) (Tonetti *et al.*, 1990), 250 and 700 times longer than the half-life of 0.017 h expected for a substance that does not offer these binding properties (Goodson, 1989). Assays for methods of detection and extraction are well developed both in buffer (Knox and

Jurand, 1979) and in physiological fluids, including blood, urine and saliva (Sharma and Bevill, 1978).

4.4.2 Encapsulation efficiencies

The encapsulation efficiency of the drug loading procedure was determined using the data obtained from dissolution tests performed on halloysite samples double drug loaded with tetracycline base. The amounts released are shown in Table 4.4.1, with their release profiles shown in Figure 4.4.1. The halloysite had been double drug loaded with tetracycline base (20 mg/ml) in a ratio of 1 ml ethanol solution to 1 g of halloysite. This gave a theoretical loading of 40 mg of tetracycline base per g of halloysite. The release studies gave a value of 15.69 mg tetracycline base per g of halloysite. This gives an encapsulation efficiency of 39.05 %. This value is greater than the value of 25.6% obtained with diltiazem HCl by Levis (2000) and is due to the second loading that was performed with the tetracycline base.

Table 4.4.1 Total amount of tetracycline base (mg) released per g of halloysite.

Sample no.	***mg of tetracycline base released per gram of halloysite***
1	15.54
2	15.54
3	15.50
4	15.25
5	15.61
6	16.72
Average	15.69

A certain amount of the drug will be lost through the drug loading process itself and through irreversible binding onto anionic sites on the halloysite. The level of irreversible binding will depend on the nature of the drug, as a cationic drug will bind

strongly to the halloysite resulting in a larger reduction of drug available for release. As tetracycline is a cationic drug, a study was performed to determine the level of irreversible binding that occurs with tetracycline due to its cationic nature. A sample of halloysite was loaded with a specified concentration of tetracycline (100 μg). A sample of this spiked halloysite was suspended in mobile phase and sonicated. The concentration of tetracycline extracted was determined by fluorescent HPLC. An average of 62.95% (S.D. 0.67) was extracted from the halloysite. This would mean that on average 37% of tetracycline available through this drug loading procedure is lost as a result of irreversible binding and the drug loading process itself.

When coating with chitosan is performed, the level of drug available for release is significantly reduced due to a number of factors. Firstly, the chitosan binding is performed by centrifugation of the halloysite sample in an acetate buffer containing chitosan. A certain amount of tetracycline will dissolve into the buffer during the process. Secondly, the chitosan will cause a further reduction in the amount available for release, as the chitosan will prevent a portion of the tetracycline from being released due to binding effects. Also the chitosan will cause an increase in the overall weight of the halloysite, reducing the tetracycline content in terms of mg tetracycline/g of coated halloysite. Encapsulation efficiencies are reduced to 32.5% (13 mg/g of coated halloysite) for tetracycline base and 41% for tetracycline HCl (16.4 mg/g of coated halloysite).

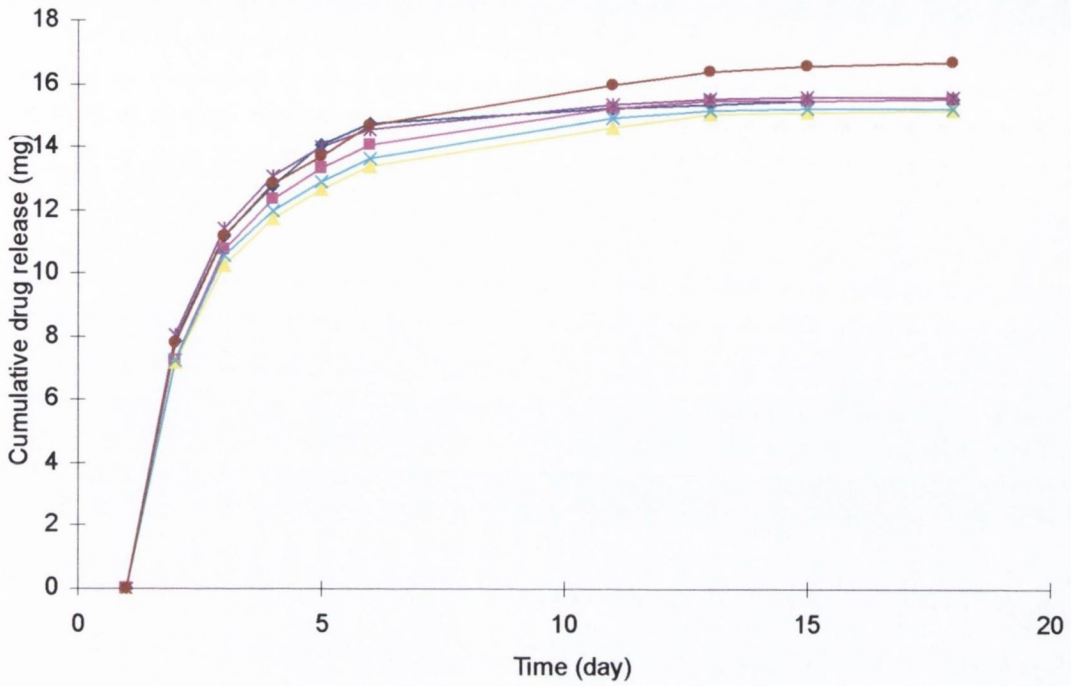


Figure 4.4.1 Cumulative amount of tetracycline base (mg) released from halloysite (1000 mg).

4.5 Impact of certain variables on the efficacy of drug loading

Halloysite was drug loaded with tetracycline base. All samples were prepared using the same method as outlined in Section 3.2. Samples were vacuumed for 30 s, mixed and the vacuum repeated. Samples were dried overnight at 50°C, and sieved through a 90 μm sieve. Samples with a double loading had the same procedure performed as for the first loading. 400 mg quantities of the drug-loaded halloysite had the drug extracted using methanol.

A 2^3 factorial study was designed in order to determine the effect of a number of variables on the efficiency of the drug loading procedure in relation to tetracycline base, which was the final drug of choice. The factors examined were:

(a) Solvent system used; ethanol or methanol: methanol due to its lower boiling point vacuums off more rapidly than ethanol, leading to much more rapid drying of the powder. It was decided to examine whether this more rapid drying affected the drug loading procedure to a significant extent.

(b) Vacuum system used: Edwards RV5 vacuum pump (UK) attached to a glass dessicator system or freeze-drying condenser (Hetosicc, Denmark) attached to an Edwards RV5 (UK) vacuum pump. The two systems used were different in the sense that the vessel and the method of vacuum application used for the drug loading were different. For the Edwards RV5 vacuum pump attached to dessicator system, a 30 ml glass jar was used to hold the sample for drug loading, whereas for the freeze drying condenser, a 100 ml round bottomed flask was used. This led to differences in the surface areas exposed for drug loading. The systems were also different in that with the dessicator system the vacuum was applied directly, whereas with the freeze-drier the vacuum was applied indirectly, via the condenser system. Unfortunately it was not possible to measure the vacuum being applied in the two systems, as this could be potentially of importance. As the vacuum pumps being used were the same, the vacuum being applied would be expected to be similar if working effectively.

(c) Single loading of halloysite *vs.* double loading of halloysite. It has been shown that repeated loading of halloysite with the drug solution can maximize the amount of drug contained in the halloysite. This is because there may not be sufficient drug in the primary loading to fill all the tubular spaces, especially in cases where the drug has relatively low solubility. Also 100% drug loading from a solution would not be expected, as evaporated solvent initially occupies loading space. As well as this, if the drug is cationic (as is the case with tetracycline) a certain amount of the primary loading may be lost, due to irreversible binding of the drug onto the anionic sites on halloysite. The

aim was to determine the effect of double loading over single loading on the final level of drug available for release.

The results obtained from the factorial analysis (Table 4.5.1) clearly demonstrate that neither the solvent type nor the vacuum apparatus used has any significant effect on the final result, in terms of amount of drug loaded. The only factor that is seen to have a significant effect is the double loading of drug over single loading, which would be expected, as increased amounts of drug are taken up into the tubules. There are no interactions observed between any of the factors. These results are interesting as they point to the drug loading procedure being a robust system where variability in the procedures used for the drug loading of halloysite does not appear to affect the final drug loading achieved.

The effect of the double drug loading over the single drug loading can be seen graphically in Figure 4.5.1. There is up to a 16-fold increase in the level of drug released from single loading to double loading. This dramatic increase can be explained by the fact that a large amount of the first loading would be irreversibly bound onto sites on the halloysite, making it unavailable for release. However when the second drug loading is performed these sites are saturated so the entire quantity of this drug loaded should enter the lumen of the halloysite and be available for release from the halloysite.

Table 4.5.1 Results from factorial analysis.

Term	*Effect*	*Coef*	*SE Coef*	*T*	*P*
solvent	0.10808	0.05404	0.03899	1.39	0.185
vacuum	0.07575	0.03787	0.03899	0.97	0.346
loading	1.34142	0.67071	0.03899	17.20	0.000
solvent* vacuum	0.06775	-0.03387	0.03899	-0.87	0.398
solvent* loading	0.03625	0.01812	0.03899	0.36	0.725
vacuum*. loading	0.2792	0.01396	0.03899	0.36	0.725
solvent* vacuum*loading	-0.06692	-0.03346	0.03899	-0.86	0.404

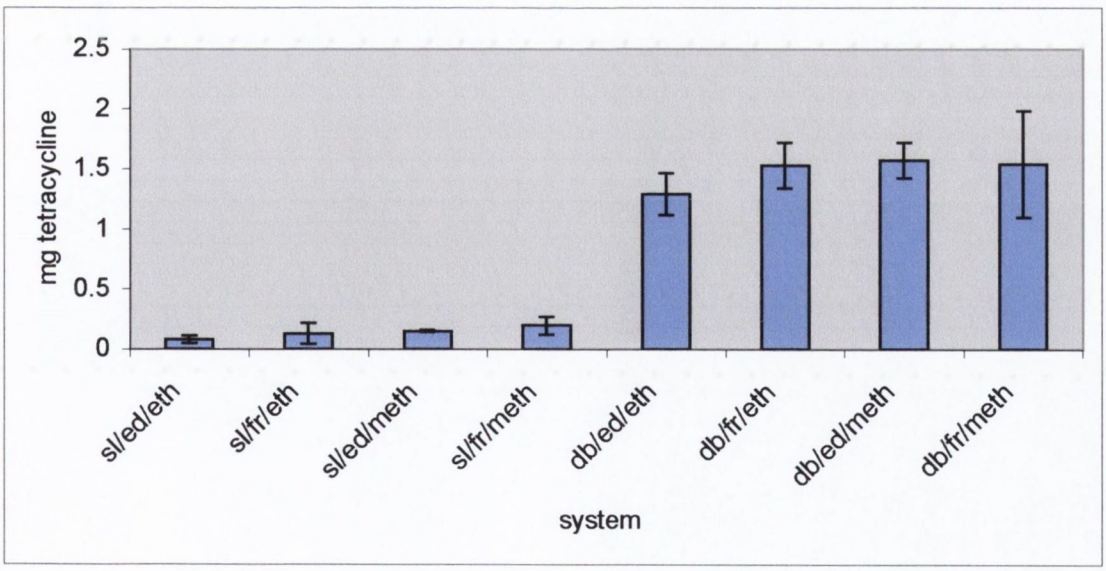


Figure 4.5.1[1] Increase in drug loaded from single loading to double loading.

[1] Loading: sl = single, db = double Solvent: eth = ethanol, meth = methanol Vacuum: fr = freeze dryer, ed = Edwards RV5

4.6 Solubility of tetracycline base and tetracycline HCl in phosphate buffer pH 6.8

The solubility of both the tetracycline base and the tetracycline hydrochloride were evaluated in phosphate buffer pH 6.8. The tetracycline base has a low solubility achieving 67% of its maximum solubility in the first 5 min. Its solubility at 120 min was 0.843 mg/ml (Figure 4.6.1). This is exactly in line with the control evaluated in a dissolution test (Figure 4.8.9). The control released an average of 1.201 mg into 1.5 ml of buffer i.e. 0.801mg/ml. This correlation between the solubility test and control test would imply that the lower volumes of buffer used in the dissolution tests are not affecting the release of the drug. Tetracycline HCl has a solubility of 3.45 mg/ml in phosphate buffer pH 6.8 (Figure 4.6.1), which is 4.09 times greater than its base. This is as expected for a hydrochloride salt. The lower solubility of the base would help improve the extended release properties of the formulation, therefore the tetracycline base was chosen as the drug to be used for the development of the final formulation.

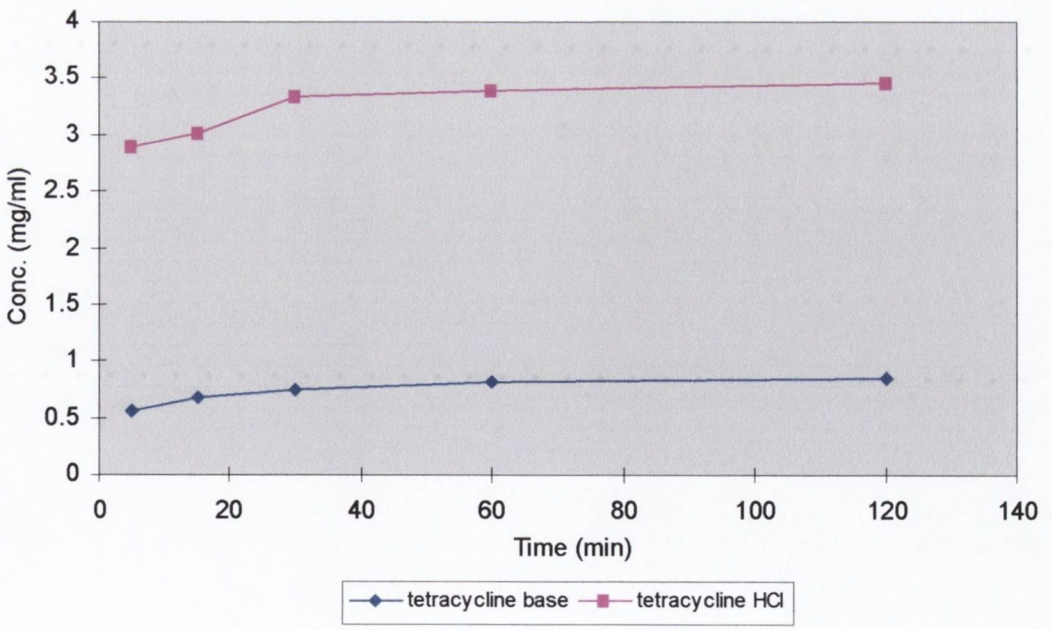


Figure 4.6.1 Solubility study on tetracycline base and tetracycline HCl in phosphate buffer pH 6.8.

4.7 Release profiles of tetracycline base and HCl

4.7.1 Introduction

The solubility of the tetracycline base and tetracycline HCl in phosphate buffer (Section 4.6) was of importance due to the nature of the subsequent dissolution test performed, where very low volumes of buffer were used. It was necessary to use these low volumes of buffer for two reasons, firstly to ensure that the levels of tetracycline released would be detectable, as only small amounts of drug are loaded into the halloysite tubules. Secondly it is necessary to create a model which would show a closer similarity to the small volume of the periodontal pocket. The periodontal pocket is a difficult physiological region to mimic. It is extremely small, with probing depths of 6-13 mm, when periodontal disease is present, and therefore the amounts of product that will be delivered to it are small. It has very low volumes of fluid flowing through it, the flow of gingival crevicular fluid is seldom greater than 10 µl/h and although these volumes of fluid are replaced frequently i.e. have a high clearance, they still only amount to volumes of the order of 1.5 ml/24 hr (Goodson, 1989). The periodontal pocket would also be considered to be a protected environment as it is difficult to access and coupled with its low volume of fluid this makes it a relatively quiescent system with little turbulence. As a result of these factors, a standard pharmacoepial dissolution test would be unsuitable as greater quantities of product or drug would have to be used to ensure detectability and large volumes of buffer in a turbulent system are used in standard BP and USP pharmacoepial dissolution tests.

4.7.2 Release from halloysite (uncoated)

Dissolution tests were performed on halloysite loaded with tetracycline base. Figure 4.7.1 shows the release of tetracycline base from halloysite. Release is relatively fast reaching 90% by day 5. Taking this into consideration, tetracycline HCl loaded halloysite was not examined, as its release would have been faster due to its higher aqueous solubility (Section 4.6). The tetracycline is stored in three different areas of

the halloysite. There is tetracycline in the lumen of the halloysite tubules, on the surface of the halloysite tubules and also between the particles of halloysite. Halloysite as is seen in Figure 4.2.1 clumps into relatively large aggregates consisting of numerous small halloysite tubules. It is likely that a certain amount of tetracycline will get trapped in these aggregates during the drug loading procedure. It is this tetracycline that will go into solution most rapidly, and therefore results in much of the burst effect that is seen in the release profiles. Figure 4.7.1 shows a 45% burst release occurring in the first 24 hr. The rest of the tetracycline base leaches relatively rapidly out of the lumens of the halloysite tubules when the halloysite is uncoated, offering a relatively poor controlled release of drug. This is due to the fact that there is no coating covering the open end of the tubules, therefore the drug can diffuse rapidly through the open pore at the end of the tubule.

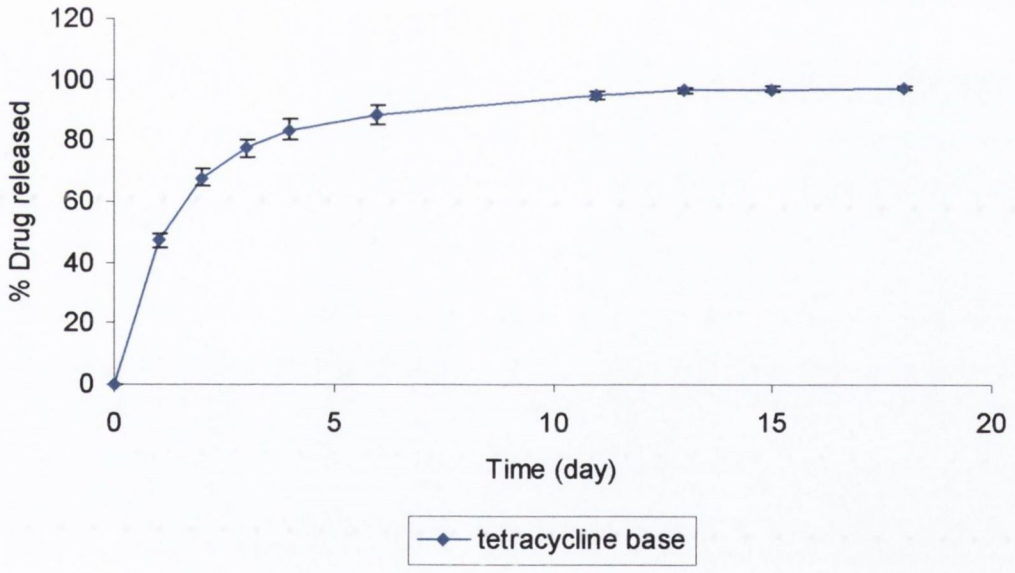


Figure 4.7.1 Release of tetracycline base from halloysite (no chitosan coating).

4.7.3 Release from halloysite coated with chitosan

Dissolution tests were performed on halloysite loaded with tetracycline base or tetracycline HCl and coated with chitosan. The release profiles of tetracycline base and tetracycline HCl loaded halloysite, coated with chitosan are shown in Figure 4.7.2. When the halloysite is coated with chitosan this greatly retards the release of the drug, with 90% release of tetracycline base occurring at day 23, a 4.5 fold increase from halloysite with no chitosan coating. The tetracycline HCl is released more quickly as would be expected taking into account its higher solubility, with 90% release at day 16. When the difference in release between tetracycline base and tetracycline HCl coated with chitosan was examined statistically at days 4, 9 and 16 the results were found to be significant. A paired t-test with a confidence interval of 95% ($p<0.05$) was performed on the results from these 3 time points and gave p-values of 0.0009, 0.04 and 0.034 for days 4, 9 and 16 respectively. After day 16 no significant difference was observed. The lower solubility of tetracycline base can therefore be considered to have a significant impact on the controlled release properties of the system, reducing the release over the first two weeks. Low levels of both tetracycline base and tetracycline HCl continued to be released until day 50 with the chitosan coated halloysite.

The release of drug is the same over the first 48-72 hr for both tetracycline base and tetracycline HCl. This drug release is most likely the portion of the drug that is loaded into the intra-tubular spaces, found in the halloysite aggregates. These will go into solution more rapidly as the aggregate breaks up in the buffer medium. This portion of the drug does not have to diffuse through the lumens of the halloysite tubules and so is not diffusion controlled to the same extent, therefore the differing solubility of the tetracycline base and tetracycline HCl will not have as significant an impact.

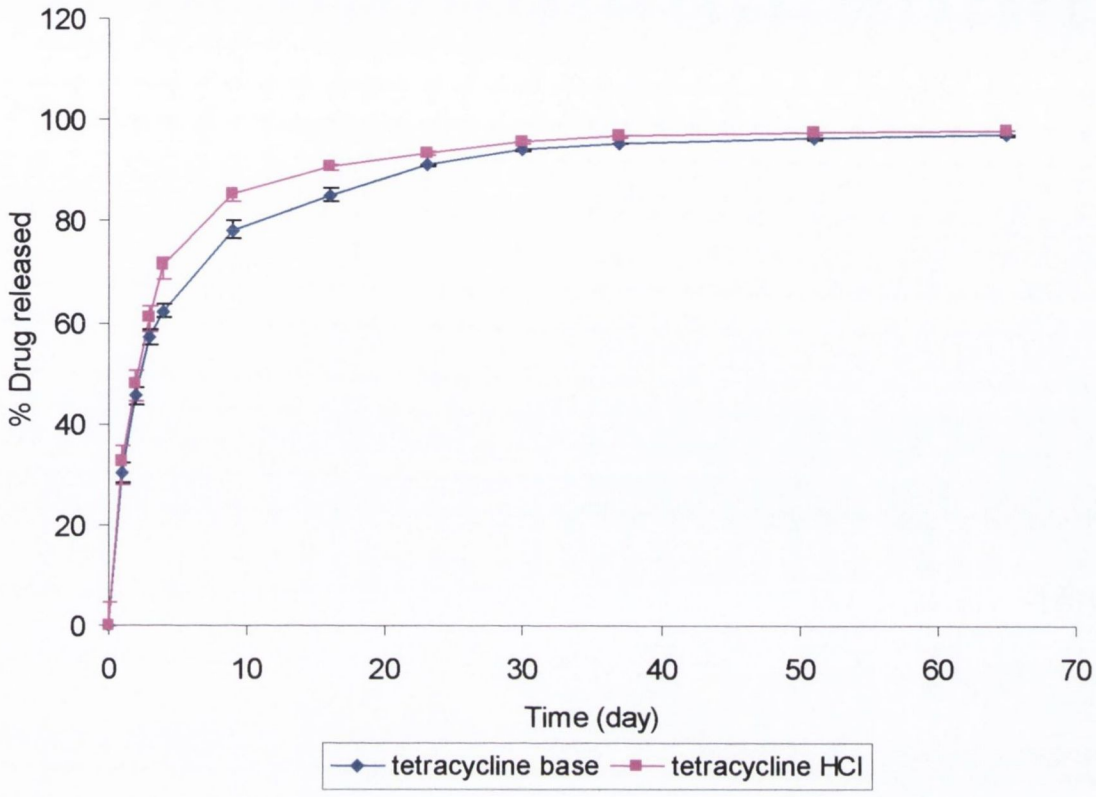


Figure 4.7.2 Release of tetracycline base and tetracycline HCl from halloysite coated with chitosan.

4.7.4 Comparison of coated and uncoated halloysite

The difference in release profiles from halloysite with and without the chitosan coating is displayed in Figure 4.7.3. Both samples show a significant burst effect, with 30% drug being released from the coated halloysite and 45% being released from the uncoated halloysite in the first 24 hr of the dissolution test. However after the initial burst release the chitosan coated halloysite showed a dramatic reduction in release in comparison to the uncoated halloysite. At day 9 the uncoated halloysite has released 88% of its final drug load while the coated halloysite has only released 78% of its total drug load. The initial burst release in both cases is most likely due to tetracycline that is on the surface of the halloysite or deposited between tubules and therefore goes into

solution rapidly when placed in the dissolution medium, compared with the drug which is within the inner lumen of the halloysite. The reduction in burst release between the coated halloysite and uncoated halloysite would point to the chitosan having an effect on either the intra-tubular drug or the drug found on the surface of the halloysite tubules. It is more likely that it is the drug on the surface of the tubules that is affected as the chitosan coating may bind over the tetracycline resulting in the presence of an extra layer that this surface tetracycline previously had not to diffuse through. The drug in the lumen of the halloysite will be released more slowly due to the fact that it must diffuse out through the pores of the halloysite, first traveling through the narrow lumen to reach the open ends of the halloysite. The release of the drug contained in the lumen of the halloysite is further retarded by the presence of the chitosan coating on the surface of the halloysite, which provide a further barrier through which the drug must diffuse. Chitosan is particularly effective at retarding drug release at neutral pH, which these dissolution test were performed at (Singh and Ray, 1999, Ganza-González *et al.*, 1999). This is due to the insoluble nature of chitosan at neutral pH, which results in excellent retention of the chitosan structure and so a prolonged ability to provide sustained release.

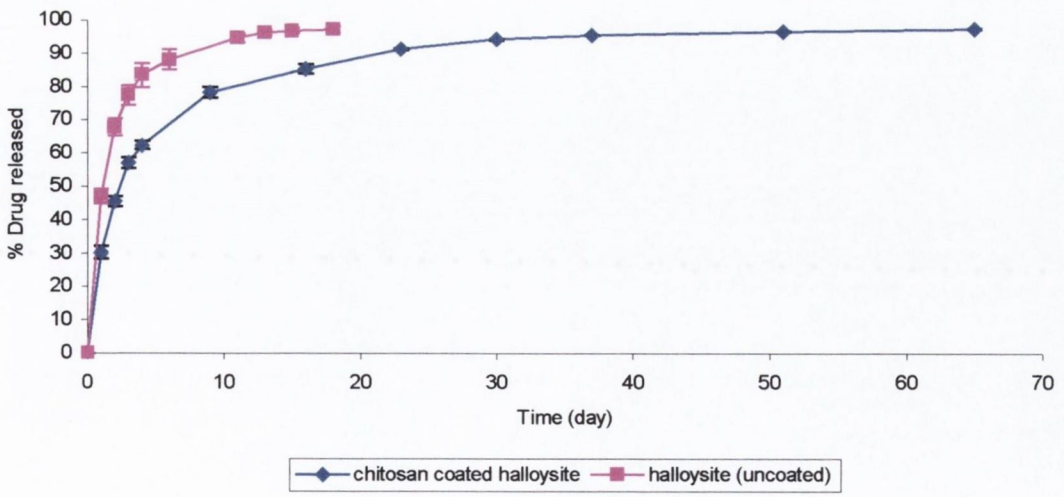


Figure 4.7.3 Release of tetracycline base from halloysite (uncoated) and halloysite coated with chitosan.

4.8 Formulation of a delivery system using a thermoresponsive polymer

4.8.1 Introduction

The halloysite loaded with tetracycline base and coated with chitosan showed a promising release profile. Therefore the next step was to develop a delivery system for the drug loaded halloysite and examine the impact that this had on the release of drug. The delivery system developed must possess the properties of ease of application and also have sufficient adherence to ensure that the product will be retained in the periodontal pocket for the period of drug release.

4.8.2 *Poloxamer 407* (Lutrol F127™)

Poloxamer 407 (*Lutrol F127™*) is a polyoxyethylene-polyoxypropylene copolymer with the chemical structure shown in Figure 4.8.1.

$$HO\,(C_2H_4O)_a\,(C_3H_6O)_b\,(C_2H_4O)_a\,H$$

Figure 4.8.1 Chemical structure of *poloxamer* copolymers.

The main characteristic of these polymers, relative to this study, is their thermoreversible nature, with aqueous *poloxamer 407* solutions being liquid below 20°C and gelling at higher temperatures, at concentrations of 20% w/w and above. These gels retain their characteristics and properties throughout repeated heating and cooling processes, with their gel strength and viscosity dependent on polymer concentration. The mechanism of action behind the thermoreversible property of the poloxamer is not fully understood. It is thought that as temperature increases micellar entanglement is promoted, leading to gel formation and an overall increase in bulk viscosity. This is thought to occur due to conformational changes in the methyl groups of the polyoxypropylene, within the hydrophobic micellar region and in the motion of the hydrophilic end chains, causing dehydration and an increase in end chain friction,

leading to gel formation (Gilbert *et al.*, 1987). The toxicity of this polymer series is largely dependent on their molecular weight. *Poloxamer 407* has an oral LD50 of > 10 g/kg and a dermal LD50 of > 5 g/kg. Topical preparations have been shown to provoke neither skin irritation or sensitivity and have been shown to be safe to use topically and intramuscularly (Sagrado *et al.*, 1994). The addition of polyethylene glycol (PEG) causes an increase in the aqueous gelation temperature of *poloxamer 407*, with the extent of the increase depending on PEG chain length and concentration (Gilbert *et al.*, 1987).

Poloxamer 407 was chosen as a possible delivery system for the drug loaded halloysite because it is a liquid at room temperature, permitting ease of syringing into the periodontal pocket, but forms a strong gel at body temperature, which should provide retention in the periodontal pocket.

4.8.3 Rheological properties of *poloxamer 407* systems

The *poloxamer 407* gel systems were characterized rheologically using their storage modulus (G') and apparent viscosity (η_{app}). The G' (Pa) is a measure of the elasticity of the system which reflects the solid-like component of the gel. Therefore the larger the storage modulus the more solid like behavior the system possesses. The more solid the system the better its retention properties should be, therefore it is desirable to have a gelling temperature of between 25°C and 30°C, so as to prevent the need for refrigerated storage, but also to have a high storage modulus at body temperature (37°C).

Two concentrations of *poloxamer 407* 25% w/w and 20% w/w were initially studied, as shown in Figure 4.8.2. The 25% w/w system showed a gelling temperature of approximately 15°C, which was considered too low, as it was preferable if the product was syringeable at room temperature, in order to avoid it having to be stored under refrigerated conditions. It was therefore decided to develop the formulation based on the 20% w/w concentration, which showed a sol-gel transition temperature of just

below 20°C. These sol-gel transition temperatures correlate well with those seen by Esposito *et al.*, (1996). The 20% w/w *poloxamer 407* showed a 30% reduction in the storage modulus at 37°C compared to the 25% w/w, however the storage modulus was still high at 18,810 Pa.

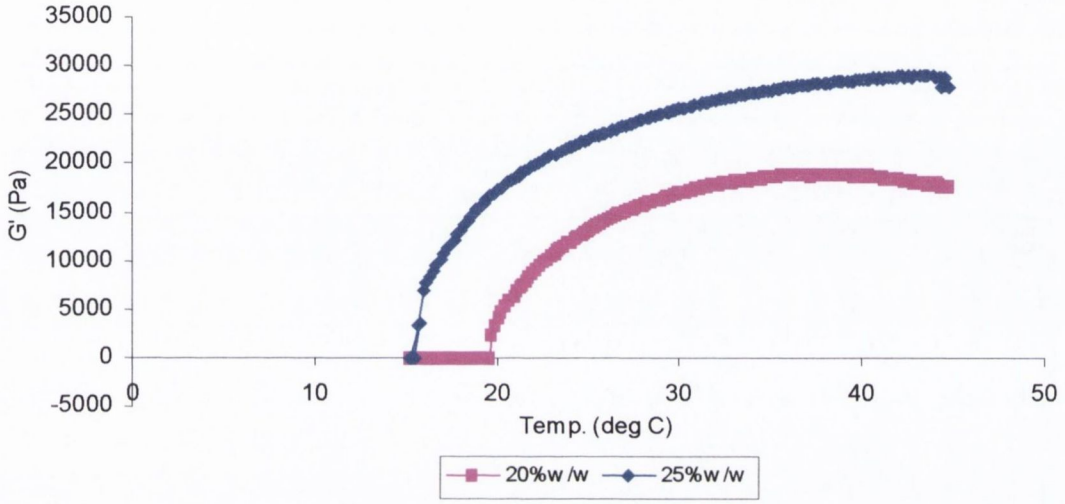


Figure 4.8.2 Storage moduli of two concentrations of *poloxamer 407*, 20% w/w and 25% w/w.

4.8.4 Effect of polyethylene glycol on the rheology of *poloxamer 407* systems

In order to increase the gelling temperature of *poloxamer 407* 20% w/w further, the addition of polyethylene glycols (PEG) was examined. Two polyethylene glycols were examined for use, PEG 6,000 and PEG 20,000. It was found that high concentrations of PEG 6,000 were required to give a small increase in gelation temperature and that the transition temperature was broadened by these high concentrations. PEG 20,000 required low concentrations to give significant changes in gelation temperatures, with the transition temperature remaining narrow (Figure 4.8.3). A concentration of PEG 20,000 0.5% w/w gave a gelation temperature of 25°C when combined with a concentration of *poloxamer 407* 20% w/w. This was considered suitable if the product was to be stored at room temperature. PEG 20,000 1% w/w addition brought the

gelation temperature to about 30°C with a broader transition and a much lower storage modulus; as a result it was considered that PEG 20,000 0.5% w/w was a better choice.

The addition of the PEG 20,000 did however cause a further reduction in the storage modulus, which is undesirable as it points to a weaker gel forming which may reduce the ability of the product to be retained in the periodontal pocket. The PEG causes an increase in the gelation temperature by modifying the micellar formation of the poloxamer molecules and possibly also forming mixed micelles with the *poloxamer* (Gilbert *et al.*, 1987). This disruption to the structure of the *poloxamer* micellar structure results in a lower storage modulus, as the PEG reduces the ability of the *poloxamer* molecules to associate with one another, so weakening the structural integrity of the system. This disruption to the structure is clearly concentration related, with the 1% PEG 20,000 causing a further decrease in the storage moduli when compared to the 0.5% PEG 20,000. At 37°C the storage modulus of the system containing 20% w/w *poloxamer 407* and 0.5% w/w PEG 20,000 was only 10,000 Pa. This is a further 45% reduction in the storage modulus of the *poloxamer 407* 20% w/w system alone.

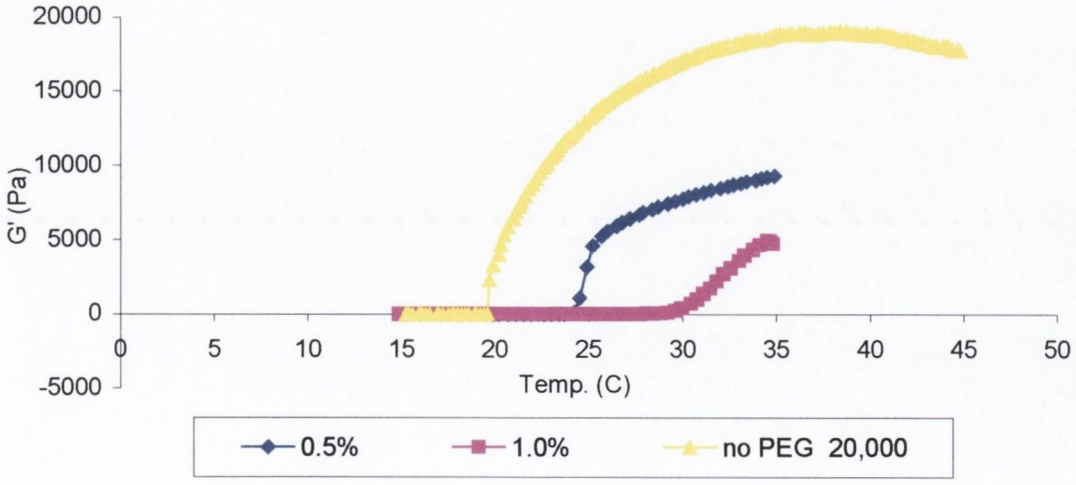


Figure 4.8.3 Storage moduli of *poloxamer 407* 20% w/w containing 0.5% w/w PEG 20,000, 1% w/w PEG 20,000 and no PEG.

4.8.5 Effect of octyl CA on the rheology of *poloxamer 407* systems

The retention of the product in the periodontal pocket was of major concern as it was necessary to ensure that the product could remain in the pocket for the entire period of the drug release from the coated halloysite i.e. a period of up to 6 weeks. It was felt that due to the significant drop in the storage modulus, as a result of the addition of the PEG 20,000, the *poloxamer 407* system might not be strong enough to ensure retention in the periodontal pocket for the required time period. To try and improve the possible retention time it was decided to include octyl CA (an alkyl-2-cyanoacrylate) in the formulation. The octyl CA is a powerful tissue adhesive which it was thought, would greatly improve retention of the product in the pocket, through its adhesive abilities. Octyl CA has been shown to be the second best tolerated of the commonly used synthetic tissue adhesives (Refojo *et al.*, 1971). Octyl CA is also currently used in the dental profession as an adhesive to hold dressings in place and this would appear to further confirm its safety (Stoller *et al.*, 1998).

Polymerization of the cyanoacrylates can be suppressed by maintaining an acidic environment. It was decided to create an acidic system incorporating the octyl CA, thereby suppressing polymerization prior to use. The theory is that the final system (halloysite double drug loaded with tetracycline base and coated with chitosan contained in the *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w and octyl CA 1% w/w, water to 100% system, pH 4) will be injected at room temperature into the periodontal pocket, where the *poloxamer 407* system will gel at the increased temperature of the periodontal pocket giving a certain degree of retention to the product. The natural buffering capacity of the periodontal pocket should over the course of time cause the gel to reach a more neutral pH, allowing polymerization of the octyl CA to occur and increasing tissue adhesion as a result, giving increased retention to the product.

To lower the pH of the poloxamer/PEG system to 4, 10% glacial acetic acid was added prior to addition of the octyl CA. Again both 20% w/w and 25% w/w *poloxamer*

systems were examined, but the 20% w/w still showed a more favorable gelation temperature with the octyl CA incorporated (Figure 4.8.4). The presence of the octyl CA affected gelation temperatures, having the effect of slightly increasing the gelation temperature of the system (Figure 4.8.4). This would imply that the octyl CA further disrupted association of the poloxamer micelles, however there was no further reduction in the storage modulus of the system. Overall it was still the concentration of PEG 20,000 0.5% w/w that gave the best results in the presence of octyl CA 1% w/w. Octyl CA 1% w/w was the concentration chosen as it was considered sufficient to provide good tissue adhesion without significantly altering any of the other properties of the system e.g. sol-gel transition temperature, storage modulus etc.

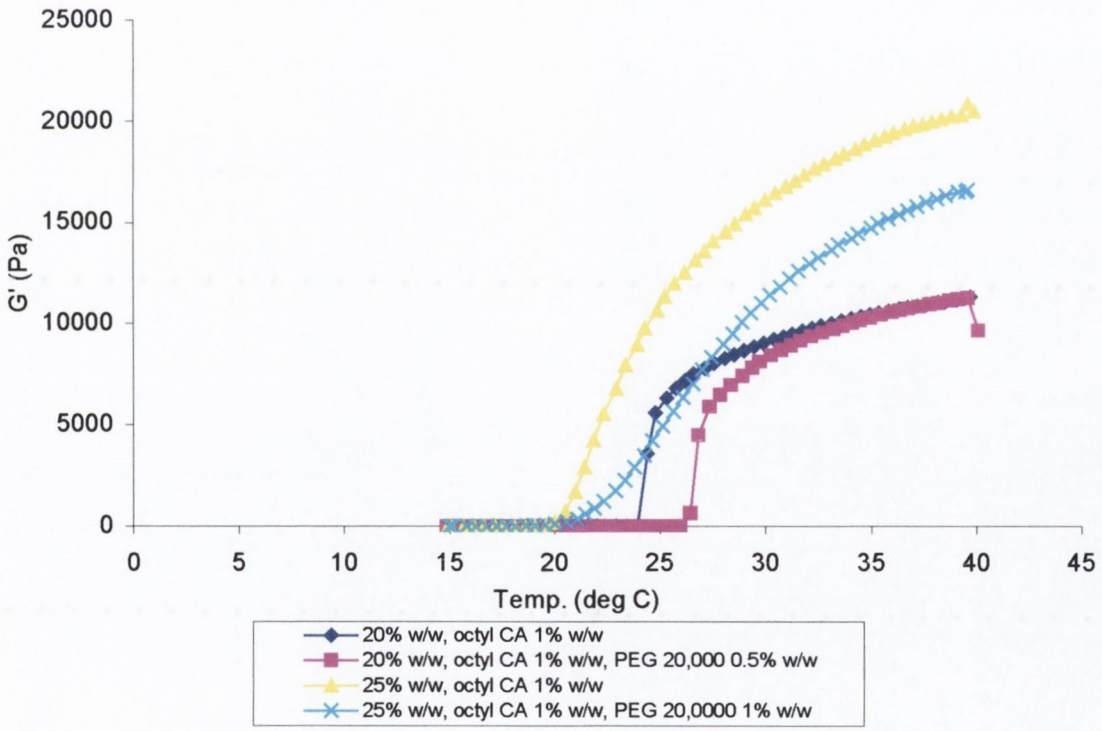


Figure 4.8.4 The effect of octyl CA on gelation temperature of *poloxamer 407* 20% w/w and 25% w/w systems.

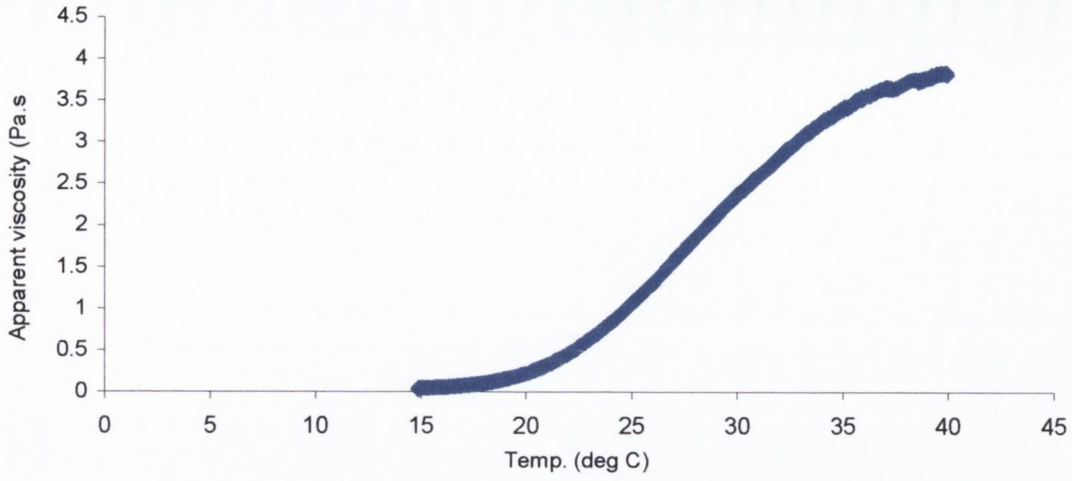


Figure 4.8.5 Apparent viscosity of *poloxamer 407* 20% w/w, octyl CA 1% w/w, PEG 20,000 0.5% w/w and water to 100%.

The apparent viscosity of this system was also examined at a constant shear rate of 75 s^{-1} over the temperature range 15-45°C (Figure 4.8.5). The apparent viscosity at 25°C was 1.06 Pa.s, but at 37°C it had increased to 3.64, which represents a 3.5 fold increase in the viscosity of the system. This is a significant increase in the apparent viscosity of the system and this physical transformation alone should help increase retention in the pocket to a certain extent. The sol-gel transition was more diffuse when examined through the viscosity of the product, with a gradual rise occurring from 20°C onwards. This is probably due to the increased micellar entanglement that occurs as the temperature increases, which will cause a gradual increase in the viscosity of the gel, but does not cause an overall structural change so is not seen in the oscillatory testing. It is only when there is an overall structural change from sol to gel that it is detected in the oscillatory testing.

4.8.6 Incorporation of halloysite and halloysite/drug into the *poloxamer* system

The final step was to incorporate the drug loaded, chitosan coated halloysite. The incorporation of the halloysite was carried out on a number of different *poloxamer* systems, as the *poloxamer 407* seemed to be highly sensitive to any added excipients.

On the addition of halloysite (not drug loaded) it was discovered that this caused a shift down in the gelation temperature, therefore the level of PEG 20,000 was increased back to 1% w/w in order to counteract this, resulting in a transition temperature of 25°C (Figure 4.8.6). The downward shift in temperature gelation on the addition of halloysite could be due to the cationic charge on the halloysite causing disruption to the micellar structure of the *poloxamer*. Possibly, it could be due to the polyoxyethylene chains binding to the halloysite causing increased entanglement of the micellar structure of the *poloxamer 407* thus leading to a decrease in gelation temperature.

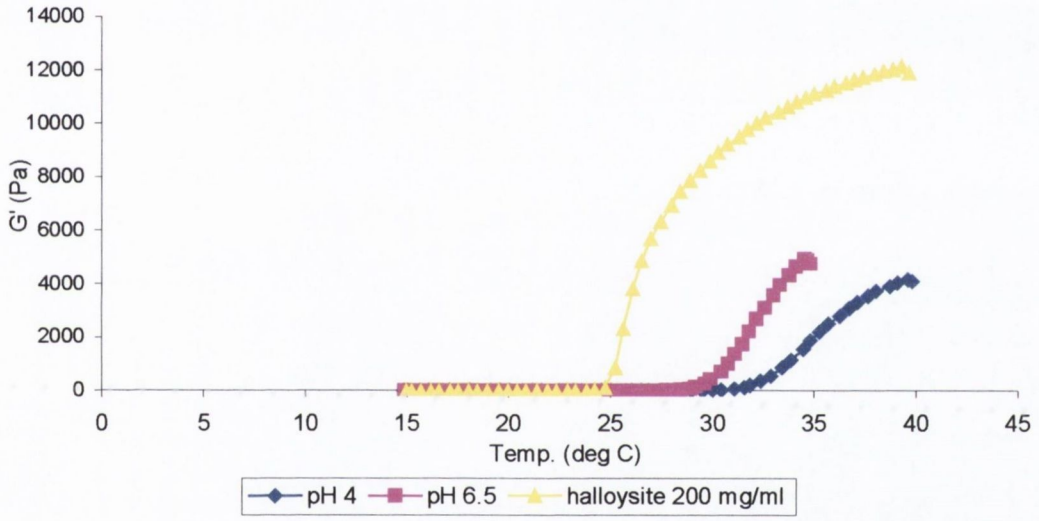


Figure 4.8.6 Effect of pH and presence of halloysite (not drug loaded) on the gelling temperature range of *poloxamer 407* 20% w/w, PEG 20,000 1% w/w.

On addition of halloysite that had been drug loaded with tetracycline base and coated with chitosan at a loading level of 200 mg/ml a thick yet syringeable liquid was obtained at room temperature for all systems. On performing a temperature ramp, significant differences were observed. The system consisting of 200 mg of halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% w/w PEG 20,000 1% w/w, octyl CA 1% w/w and water to 100%, lost nearly all thermoresponsivity, while the system without PEG 20,000 showed a sharp transition

but at a lower temperature (21.9°C) (Figure 4.8.7). The system consisting of 200 mg of halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w and water to 100%, pH 4, showed a broader transition at a slightly higher temperature (23°C) (Figure 4.8.7). The tetracycline has the effect of further decreasing the gelation temperature, which is similar to the result seen in Esposito *et al.* (1996). This effect could possibly be related to the presence of the tetracycline on the surface of the halloysite, which could further act to disrupt the micellar structure, increasing entanglement and further reducing the gelation temperature. The effect of the broader transition is significant as the storage modulus of the *poloxamer 407* 20% w/w, octyl CA 1% w/w water to 100%, system reaches 6288 Pa at 21.9°C, whereas the storage modulus of the poloxamer 20% w/w, octyl CA 1% w/w, PEG 20,000 0.5% w/w, water to 100%, only reaches 5117 Pa at 25.17°C. This should allow the syringeability to be sufficient up to a temperature of 25.17°C. At 37°C the storage modulus of this system is 14480 Pa, which is significantly higher than the system minus the drug component. This is advantageous, as it should aid better retention in the periodontal pocket.

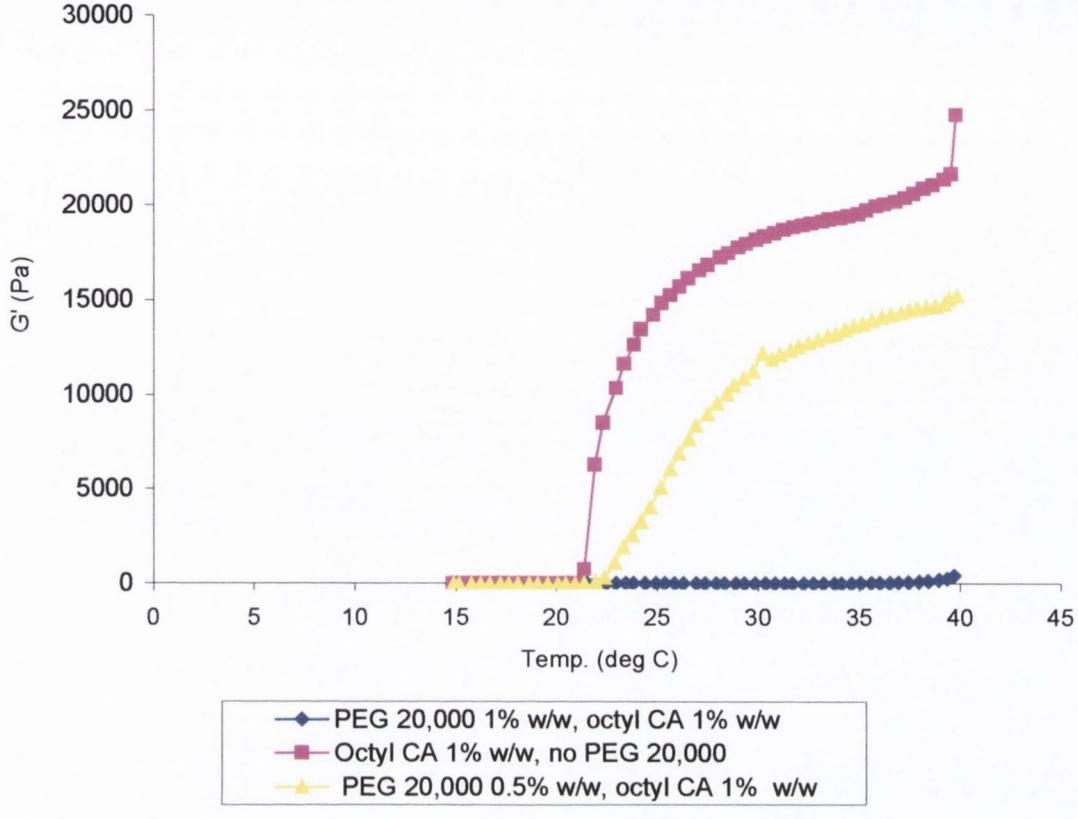


Figure 4.8.7 Effect of addition of 200 mg halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% w/w systems on storage modulus and gelling temperature.

The final system (200 mg of halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w and water to 100%, pH 4) was then run for 6 hr at 37°C to examine the effect of time on polymerization of the octyl CA (Figure 4.8.8). A solvent trap was used to prevent dehydration of the sample occurring over time. When the sample was placed on the rheometer the pH of the system was 4. At this pH the polymerization of the octyl CA is largely suppressed. However on leaving the sample exposed for a number of hours polymerization of the octyl CA would appear to start to occur as over the course of six hours the storage modulus doubles, going from 17,000 Pa to 35,000 Pa.

This indicates the increasing gel strength of the system, which should increase the retentive ability of the product in the periodontal pocket.

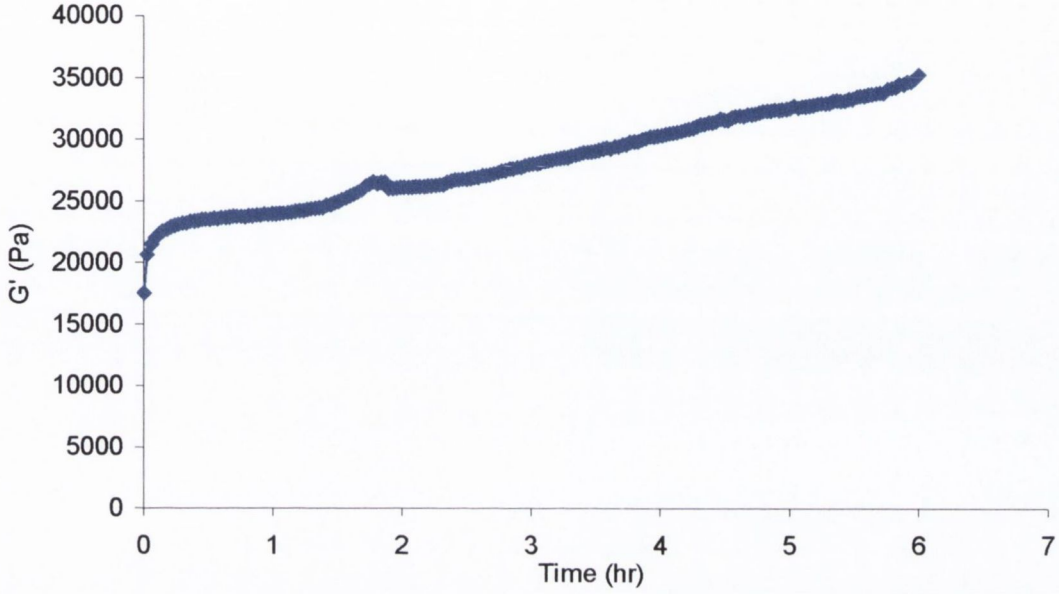


Figure 4.8.8 Change in storage modulus over time of final formulation.

This result could potentially explain a problem that was experienced during the development of the product. When batches of the same product were tested, while there gelling temperatures remained consistent their storage moduli showed significant differences. This can be explained by the above results, as the sample would have been run at different times, up to 24 hours, after preparation. To resolve this problem samples need to be put into syringes as soon as the samples are prepared in order to ensure that the systems remain at their pH of 4, which reduces the rate of polymerization of the octyl CA. These results would also initially suggest that polymerization of the octyl CA does not affect the gelation temperature of the system.

4.8.7 Release of tetracycline base from the final formulation developed

On determining the system that met the requirements of syringeability and good retention, it was necessary to determine the effect this system would have on the drug release. A dissolution test was performed in order to examine this. It was found as can be observed in Figure 4.8.8 that the presence of the gel system (*poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w, water to 100%) had no effect on the drug release, with the release profile being identical to the one obtained without the *poloxamer* delivery system. Figure 4.8.8 also gives an indication of the impact that the sustained delivery system has on the release profile, with the benefits of the developed final system clearly seen. The halloysite loaded with tetracycline base shows controlled release properties when compared to the free drug alone, on the addition of the chitosan coating this controlled release is significantly improved. The total release for the 63-day period was 386 μg tetracycline base released from 250 mg of final product and this was achieved in 24 hr with the pure drug, which acted as a control (Figure 4.8.9).

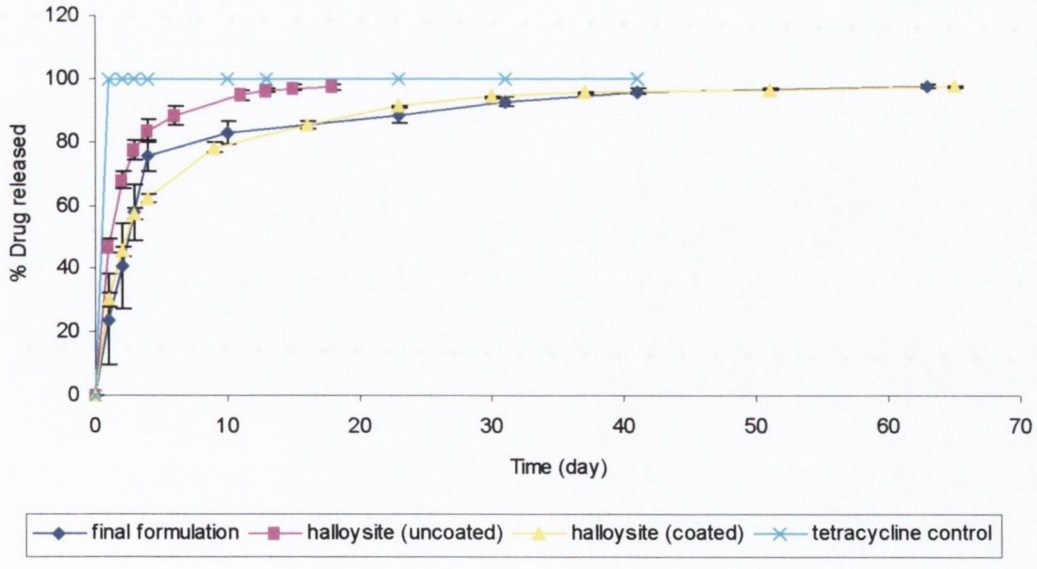


Figure 4.8.9 Release profiles for tetracycline base control, halloysite coated and uncoated, and the final formulation.

The spread of the total release is shown in Figure 4.8.10. The burst effect at the beginning of the release profile would have the impact of reducing bacterial counts significantly in the first week. Initial concentrations released over days 1 and 2 were several orders of magnitude greater than the minimum inhibitory concentration (MIC) for certain periodontopathic bacteria (Table 4.8.1). Mean bactericidal concentrations (MBC) were achieved for the first two days of release, however they were not maintained for the duration of the drug release. The high levels of the first 48 hr could have a significant impact on bacterial levels, due to the fact that bactericidal levels were achieved, consequent concentrations were adequately high to achieve suppression of *P. gingivalis* and *P. intermedia* until day 40.

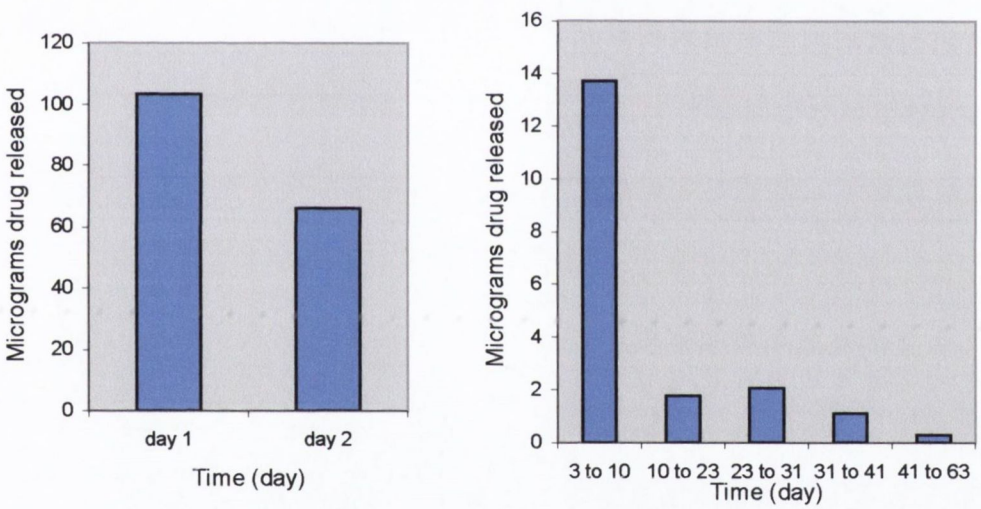


Figure 4.8.10 Cumulative release over time intervals indicated, of tetracycline base (μg) from final formulation.

Table 4.8.1 MIC (90%) and MBC (90%) values for tetracycline against certain periodontopathic bacteria (adapted from Miyake *et al.*, 1995).

	MIC (μg/ml)	***MBC (μg/ml)***
	90%	90%
P. gingivalis	0.5	16
P. intermedia	0.25	0.5
A. actinomycetecomitans	4	>64

However it is difficult to tell whether this release profile will mirror the *in vivo* release profile for a number of reasons. Tetracycline is known to show a good ability to bind to tissue *in vivo* and therefore it can be expected that a certain amount of the tetracycline will bind to tissue and tooth enamel in the periodontal pocket. As levels of tetracycline in the pocket fall this tetracycline could be released into the pocket, boosting levels of tetracycline at a later stage. Also due to the extremely low flow (10 μl/hr) and the poor solubility of tetracycline base in aqueous environments it is possible that a significant concentration of antibiotic will accumulate in the pocket. This could have the effect of preventing the further release of antibiotic from the drug delivery system until concentrations begin to fall. This will result in a more uniform concentration of tetracycline being maintained in the periodontal pocket for a more prolonged period of time. If these propositions are the case a far more uniform release over time should be observed. Therefore until *in vivo* studies are carried out in the periodontal pocket, it is difficult to draw conclusions as to whether sufficient levels of antibiotic will be present to suppress periodontopathic bacteria for an extended duration of time.

4.9 Bioadhesion of final formulation

A bioadhesion study was performed on the final formulation (200 mg of halloysite double loaded with tetracycline base, coated with chitosan per ml of *poloxamer 407*

20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w, water to 100%, pH 4) in order to determine the adhesiveness of the system to mucosal tissue. FPOM was used as the bioadhesion model. Due to small amounts of this being available only one time point was examined. The poloxamer system consisting of *poloxamer 407* 20% w/w, PEG 20,000 0.5%, octyl CA 1%, water to 100%, pH 4 and the final system as above were tested.

Figure 4.9.1 shows the results of the bioadhesion studies. System 1 is obviously highly bioadhesive as expected with octyl CA being a powerful tissue adhesive. The addition of the halloysite component gives an increase of approximately 10% to both the peak detachment force and the work of adhesion. This could be due to the presence of the chitosan, which is a bioadhesive material in itself (Hassan and Gallo, 1990). The strong bioadhesive force present will help anchor the product in the periodontal pocket after injection, providing good retention. Strong retention of the product in the periodontal pocket is essential in order that it remains in place for the entire period of the drug release.

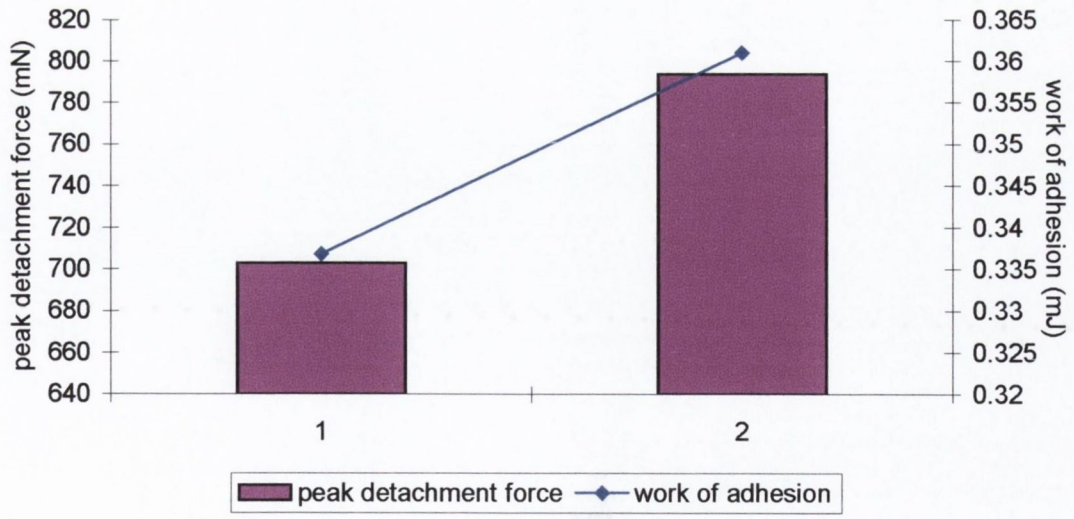


Figure 4.9.1 1. *Poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w, pH 4

2. Final formulation

4.10 Syringeability of *poloxamer 407* systems

Syringeability of various *poloxamer 407* systems was examined to determine the effect that both temperature and the addition of excipients have on the force required to expel the product. This is of importance as the product must be able to be delivered by syringe through a needle, in order to fulfill the requirement of ease of application. The systems examined for syringeability are listed in Table 4.10.1.

The effect temperature has on the syringeability of the system can be clearly seen in Figure 4.10.1. System 1 has a syringeability of 21.68 N at 4°C and 185.28 N at 20°C, which is an 8.5 fold increase in syringeability seen over a 5 fold increase in temperature (°C). Concentration also has a significant impact with syringeability increasing from 21.68 N to 41.77 N with increasing concentration from 20% w/w to 25% w/w at 4°C. The syringeability of 25% w/w poloxamer at 20°C could not be determined due to the solid nature of the polymer at this concentration and temperature.

Table 4.10.1 Systems examined for syringeability

System no.	***System***
1.	*poloxamer 407* 20% w/w
2.	*poloxamer 407* 25% w/w
3.	*poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w
4.	*poloxamer 407* 20% w/w, PEG 0.5% w/w, octyl CA 1% w/w
5.	*poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w and 200 mg/ml tetracycline base loaded chitosan coated halloysite

On addition of the PEG 20,000 at a concentration of 0.5% w/w to the *poloxamer 407* 20% w/w, the syringeability of the system at 20°C is almost halved (Figure 4.10.1). This would correlate well with the increase in gelling temperature seen with the addition of PEG 20,000 0.5% w/w. The system incorporating the octyl CA shows an interesting result with syringeability being almost the same at both 4°C and 20°C. This is clearly due to the effect that both PEG 20,000 and octyl CA have on the gelling temperature and as a result, the viscosity, of the system. The combination of these two components significantly increases the gelling transition temperature of the system, meaning that the apparent viscosity of the system does not begin to rise until higher temperatures have been reached. The addition of tetracycline base loaded halloysite does not change this syringeability at 20°C (Figure 4.10.1). The final syringeability shown for system 5 at 20°C is only a quarter of the syringeability of system 1 at 20°C and only double the syringeability of system 1 at 4°C, which is very desirable having regard to ease of potential administration.

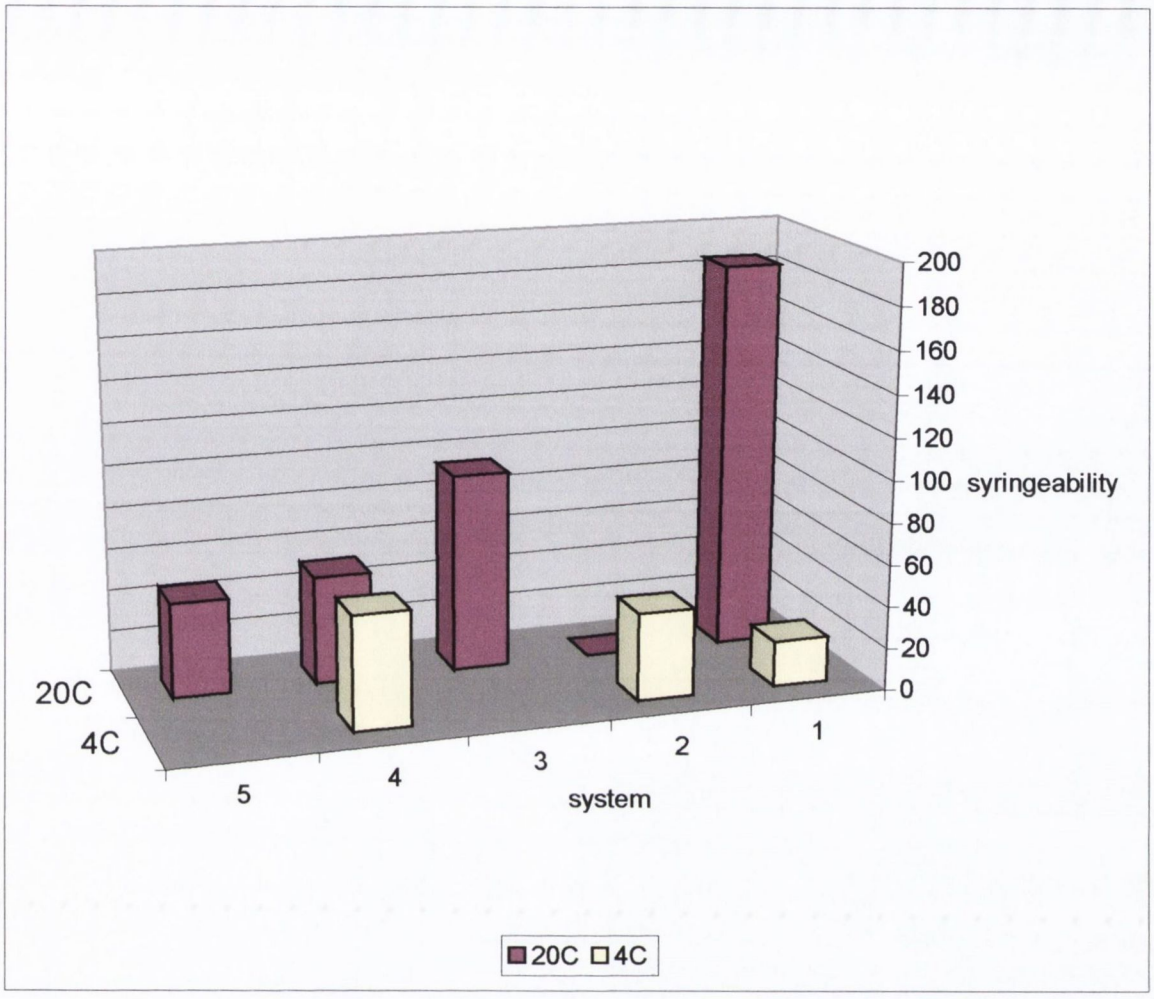


Figure 4.10.1 Syringeability of various systems (see Table 4.10.1 for details of composition) at 4°C and 20°C.

4.11 Stability study

A stability study was performed over 9 months to determine the effect time had on the syringeability, apparent viscosity and thermoresponsivity of two systems at two different temperatures. The two systems consisted of the final formulation and the gel system contained in the final formulation (Table 4.11.1). The reason for examining the gel system alone was that in the case of poor stability results from the final formulation

it would be possible to have an indication as to whether the system could be formulated as a two-component formulation that could be mixed immediately prior to application.

Table 4.11.1 Systems examined in stability study.

Label	***System***	***Storage temperature (°C)***
A	200 mg halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w, water to 100%, pH 4	25°C
B	200 mg halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w, water to 100%, pH 4	4°C
C	*poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w, water to 100%, pH 4	25°C
D	*poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w, water to 100%, pH 4	4°C

4.11.1 Visual observation

Table 4.11.2 Visual appearance of samples at various time intervals.

	System A	***System B***	***System C***	***System D***
0	Pale brown viscous system	Pale brown viscous sytem	Transparent white, gel.	Transparent, white gel
1 month	Brown viscous system, with no separation	Two layered system, upper layer clear fluid, lower layer a brown particulate system	Transparent, white, gel.	Transparent, white gel
3 month	Brown viscous system, with no separation	Two layered system as at 1 month	White gel, becoming more opaque	Transparent, white gel
6 month	Brown viscous system, with no separation	Same as at 1 month	White gel, opaque, with white particulate matter present, possibly microbial growth	Transparent, white gel
9 month	Brown viscous system, with no separation	Same as at 1 month	White gel, opaque, with white particulate matter present, possibly microbial growth	Transparent, white gel

System B separated rapidly on storage into its two components, halloysite and the *poloxamer 407* system, and proved to be extremely difficult to reconstitute. This means that if system A were to prove unstable, system B would not be viable as a product, as it is too difficult to mix. Therefore the product would have to be prepared as a two-component system. System C showed microbial growth after 6 months. This would mean that if the product had to be stored as two separate components it would be necessary to include a preservative in the *poloxamer 407* system, if it were to be stored at room temperature. There did not appear to be any microbial growth in system A, due to the presence of the antibiotic tetracycline.

4.11.2 Syringeability of product over time

Figure 4.11.1shows that at time 0 the two systems that were to be examined had similar areas under the curve i.e. similar force of expulsion, but the system containing halloysite had a larger standard deviation than the *poloxamer* system. This correlated well with the syringeability data in Section 4.10. At the end of the nine month study a number of conclusions can be drawn regarding the syringeability of the various systems. Firstly the presence of the halloysite in the *poloxamer* system has no significant impact on the syringeability of the system, with all 4 systems showing approximately equal syringeability (Figure 4.11.1). Secondly a time period of nine months appears to have no impact on the syringeability of the four different systems (Figure 4.11.2), with the systems showing similar syringeability values at 9 months as was seen at time 0. While there was a larger increase in system A at 9 months, there was also a significant increase in the standard deviation, and when a t-test was performed no difference was observed between the syringeability of system A at 6 months and 9 months ($p=0.891$).

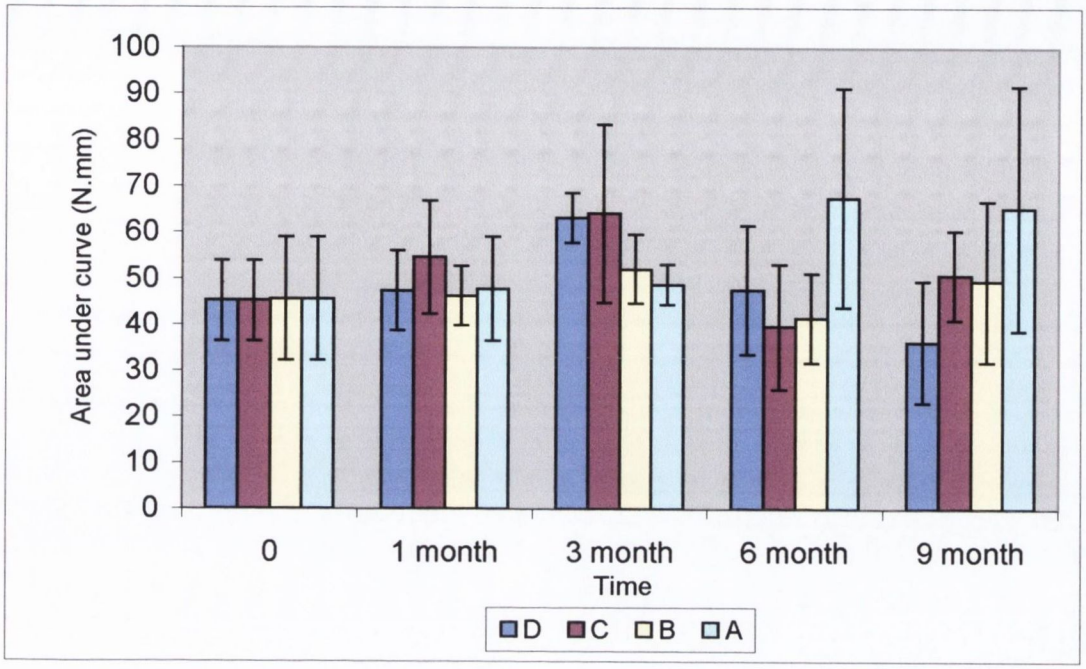


Figure 4.11.1 Changes in syringeability of each system over time.

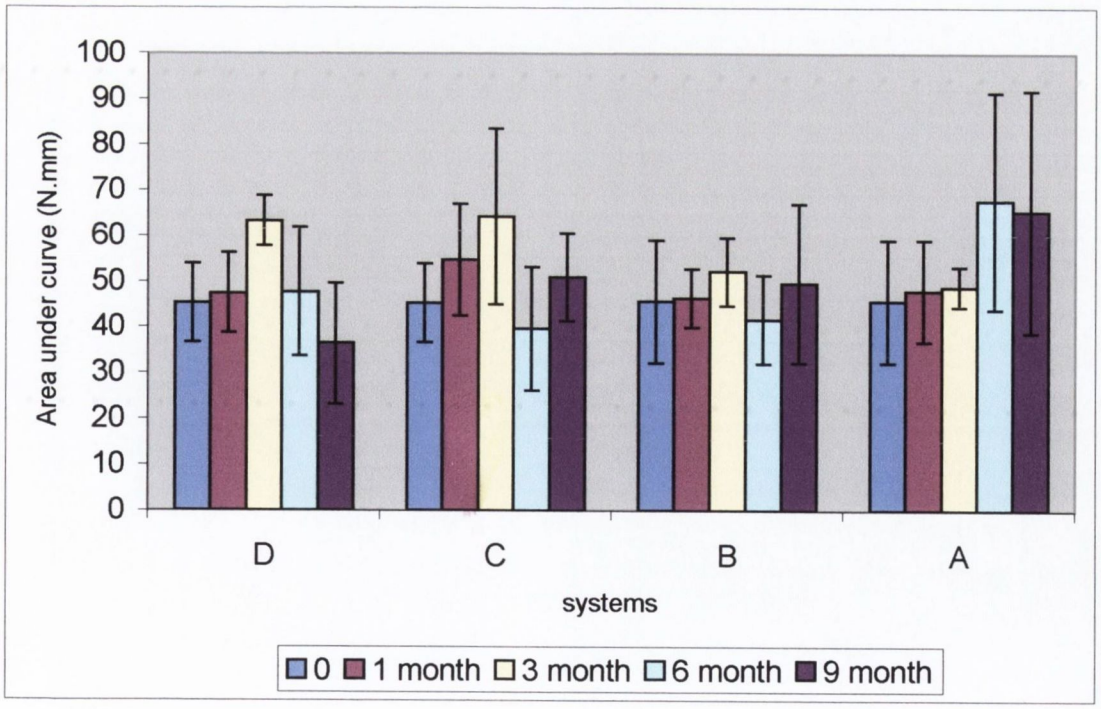


Figure 4.11.2 Comparison of syringeability of various systems over time.

4.11.3 Changes in apparent viscosity over time

The apparent viscosity of systems A/B and C/D show correlating results over the 9 month period, with apparent viscosity increasing over time in all systems. In all cases the increase was greater in systems stored at 25°C than those stored at 4°C. The increase in apparent viscosity appears to be temperature dependent with the rise occurring at higher temperatures, usually greater than 23-25°C. This accounts for the fact that while there is an increase in apparent viscosity there is not a subsequent increase in syringeability, as syringeability testing was performed at 20°C. At this temperature there was no change in apparent viscosity over time. The increase in apparent viscosity over time could be due to the octyl CA beginning to polymerize.

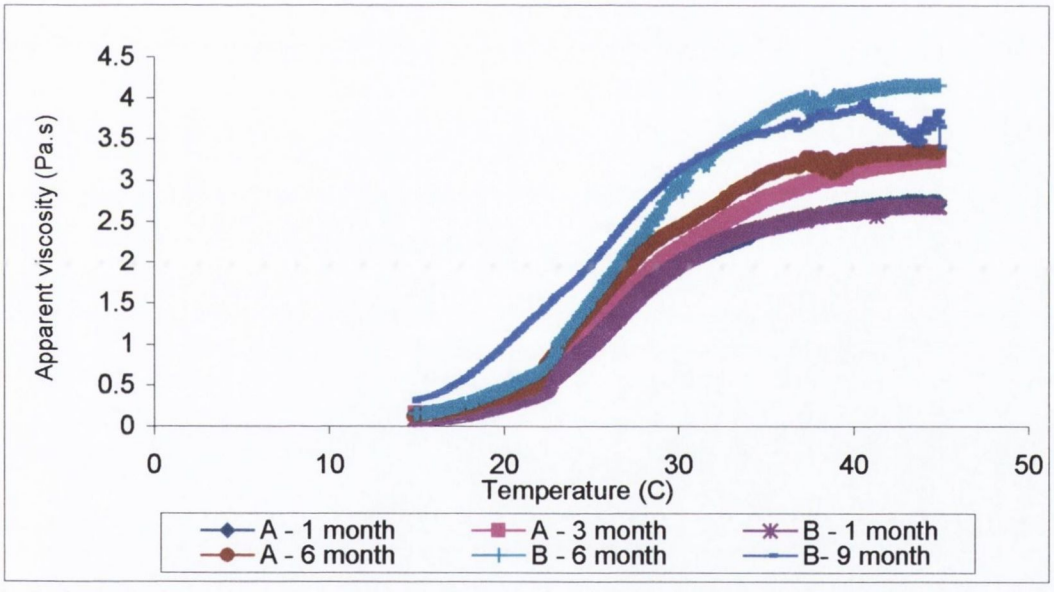


Figure 4.11.3 Changes in apparent viscosity over time of systems A and B.

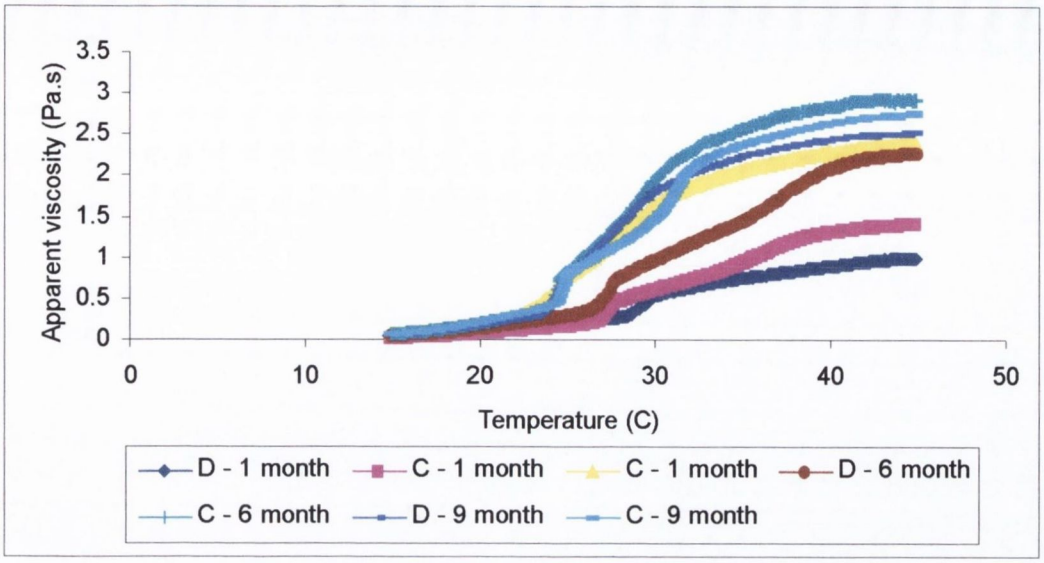


Figure 4.11.4 Changes in apparent viscosity over time in systems C and D.

4.11.4 Changes in sol-gel transition temperature over time

It is apparent from Table 4.11.3 that there is no significant change in gelling temperatures over time, with systems A and B consistently showing gelling temperatures 3-5°C below those seen for systems C and D.

Table 4.11.3 Gelling temperatures for systems over time

Time	*System A*	*System B*	*System C*	*System D*
0	24.9	24.9	31.8	31.8
1 month	27.1	25.6	27.7	25.2
3 months	26.1	24.2	31.3	34.7
6 months	Sample lost	25.7	28	31.8
9 months	24.2	28.4	29.9	28.3

The results from the stability study are extremely positive as they point to system A being suitable for storage for up to a period of 9 months. System A was the preferable system as it consisted of the final product stored at room temperature. This would mean that the product does not require specialized storage e.g. refrigeration nor does it require any pre-formulation prior to using. Also because it is stored at a temperature where the system is quite viscous, separation of the product does not occur. This is of considerable importance in order to ensure a uniform delivery of the product.

4.12 Conclusions

The aim of this work was to develop a localized drug delivery system for the treatment of periodontitis. The product was developed using the novel excipient halloysite, which was drug loaded using tetracycline base. This alone did not offer sufficient extended drug release and therefore chitosan was used as a coating on the halloysite to further retard release of drug. This resulted in a drug release over a satisfactory time period of 60 days approx.

A delivery system was required to allow for application of the halloysite to the periodontal pocket. A thermoresponsive delivery system was chosen as it offered ease of application to the periodontal pocket, with subsequent good retention at body temperature (37°C). The sol-gel transition of the thermoresponsive polymer was manipulated, using PEG 20,000 to give a suitable transition temperature. In order to improve the retention of the product further, a tissue adhesive was incorporated into the formulation. The presence of the gel system was shown to have no impact on the release profile of the system.

The bioadhesion of the final formulation was examined and it was shown to possess strong bioadhesive properties, which are necessary to ensure sufficient retention in the periodontal pocket. Syringeability of the product was also examined and it was seen to have good syringeability offering ease of application. Stability studies showed that the final formulation was stable for up to a period of 9 months, when stored at 25°C.

Chapter 5

In vivo testing of periodontitis product

5.1 *In vivo* study of local drug delivery system for the treatment of periodontitis

5.1.1 Introduction

Periodontitis is a major cause of adult tooth loss. It is an infection caused by pathogenic flora within the gingival sulcus, which as a result deepens to form a periodontal pocket (Roskos *et al.*, 1995). Traditionally, the treatment of periodontitis has consisted of mechanical debridement of supra and sub gingival plaque through root scaling and planing. This method of treatment is still widely used in patients responsive to such methods. However there are a group of patients who are less responsive to mechanical methods e.g. refractory periodontitis, rapidly progressive periodontitis and localized juvenile periodontitis. In these cases antibiotics have been used to treat periodontitis. The systemic use of antibiotics is one choice available, with the main regimens used being amoxycillin with clavulanic acid (Winkel *et al.*, 1999) and/or metronidazole, (Lopéz *et al.*, 2000, Winkel *et al.*, 2001), or tetracycline (Rams and Keyes, 1983, Haffajee *et al.*, 1988). However, regimens including clindamycin, doxycycline and azithromycin (Sigusch *et al.*, 2001, Walker and Gordon, 1990, Blandizzi *et al.*, 1999) have also been considered. The problem with systemic antibiotics is that concentrations achieved in the gingival pockets are low, considering the doses being given (Sakellari *et al.*, 2000) and compliance is a problem. As a result the localized delivery of antibiotics has became an alternative option, as much higher concentrations of antibiotics in the periodontal pocket can be achieved, without concomitant side effects (Tonetti *et al.*, 1990). Also as the product is applied directly to the periodontal pocket compliance is no longer an issue, with the delivery of the drug being removed from the patient's control. Many products are currently available on the commercial market e.g. *Actisite*™, *Periochip*™, however none are ideal. An improved delivery system was developed in this project, using the novel excipient halloysite to control release of tetracycline into the periodontal pocket. A thermoresponsive gel delivery system was created to improve ease of application and product retention in the pocket. *In vitro* studies showed it to be a promising formulation in terms of its controlled release, ease of application and good bioadhesive properties, which will aid in

retention of the product in the periodontal pocket. An *in vivo* study as described in this Chapter, was performed to evaluate the product's potential for treatment of periodontitis.

5.1.2 Aims of the *in vivo* study

In vivo properties of the product developed were examined using a wound pocket creation model to mimic periodontitis in two Labrador dogs. The testing of the product in dogs was necessary as the formulation contained the novel excipient halloysite, which is an excipient currently not approved for use in humans. However as it is chemically similar to kaolin, which is extensively used in the pharmaceutical industry, it is considered that approval for use in humans could easily be obtained. The principal information that was required from the *in vivo* study consisted of answers to the two following questions:

(a) Was retention of the product sufficient to ensure that it remained in the periodontal pocket for an adequate period of time? This fact was to be established by determining over what time period levels of tetracycline could be detected in the artificially created pockets.

(b) Was sufficient drug released to suppress microbiological activity within the pocket for an extended time period? This was to be determined by examining total CFU's under both aerobic and anaerobic conditions to determine whether numbers were suppressed in the test pockets.

5.2 Concentration of tetracycline base present in the test samples

The test system (final formulation) used in the *in vivo* study consisted of 200 mg of halloysite double loaded with tetracycline base and coated with chitosan per 1 ml of *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl CA 1% w/w and water to 100%, pH 4. The control system consisted of 200 mg/ml halloysite coated with chitosan per 1 ml of *poloxamer 407* 20% w/w, PEG 20,000 0.5% w/w, octyl cyanoacrylate 1% w/w and water to 100%, pH 4. After the test systems had been

injected into the pockets on the dogs' teeth, samples remaining from each syringe were analyzed to determine the content of tetracycline base in the test samples. The results are listed in Table 5.2.1. The average content of tetracycline base per 100 mg of product was 94.71 μg, with a standard deviation of 19.26 μg. This standard deviation is 20% of the content and represents a large spread of variation among the samples. This could be due to uneven mixing as a result of sedimentation of the halloysite fraction within the product. The sedimentation occurs as a result of the aggregated nature of halloysite (Figure 4.2.1). At low temperatures the *poloxamer 407* system has a low viscosity, due to its thermoresponsive nature, and the aggregated halloysite particles tend not to remain evenly suspended. It was found that it was difficult to achieve more uniform mixing of the sample after sedimentation had occurred, despite vigorous agitation. For the *in vivo* trials, the samples were stored at 4°C and were only removed immediately prior to application, due to concerns over the stability of the *poloxamer 407* system at higher temperatures. However a stability study subsequently performed has shown that storage at 25°C has no effect on the system and is in fact preferable, as separation of the product does not occur making it a more uniform system when being injected.

The results in Table 5.2.1 give a determination of the amount of tetracycline base present per 100 mg of product and so should give an approximate idea of the level of tetracycline that is being delivered into the periodontal pocket. However there was a problem with determining the absolute quantity of product that was delivered to the pockets in the dogs. While the weight of all syringes was taken before and after injection, there was considerable waste in some of the injection processes. As a result it is difficult to determine the exact amount of product that is administered to each pocket. The average value of the weights expressed from the syringes, as can be seen in Appendix 7, was 0.138 g. This would give an average administered dose of 130.98 ± 26.2 μg tetracycline base into each pocket, assuming no waste.

Table 5.2.1 Tetracycline base present per 100 mg of test sample (refer to Table 3.7.1 for key to pockets).

Sample no.	***Dog no.***	***Pocket***	***µg tetracycline base /100 mg of product***
1	1	URP2	113.95
2	1	LRP2	94.18
3	1	LRC	95.78
4	1	LRP4	123.77
5	1	URC	123.31
6	1	URP4	97.99
7	2	LLC	92.56
8	2	LLP2	80.77
9	2	LLP4	101.64
10	2	ULC	72.83
11	2	ULP2	63.72
12	2	ULP4	76.00
Average			***94.71***
Standard deviation			*19.26*

5.3 Release profile of tetracycline base determined from residue studies

5.3.1 Average release from test pockets

The average concentration of tetracycline base (ng) in the pockets per mg of residue is shown in Figure 5.3.1. These concentrations are a combination of test pockets in dogs 1 and 2 and control pockets in dogs 1 and 2. At day 14 there is an average concentration of 215.5 ng/mg (21.55 µg/100 mg), with levels rising slightly to 223.69 ng/mg (22.37 µg/100 mg) by day 28. By day 42 concentrations were still at 198.06 ng/mg (19.81 µg/100 mg), however by day 56 there were no detectable

levels of tetracycline remaining in the pockets of either dog 1 or dog 2. This gives an average pocket concentration of 21.24 μg/100 mg, over a 6-week period.

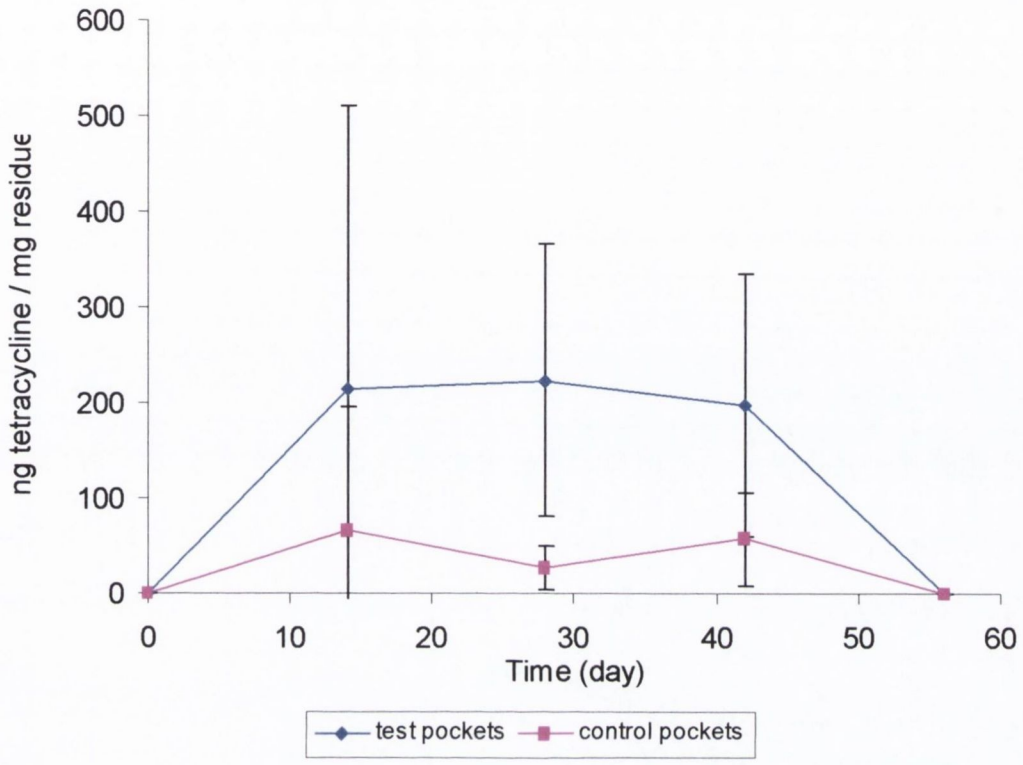


Figure 5.3.1 Concentrations of tetracycline base ng/mg of residue in test pockets.

The release profile seen in the test pockets is clearly different than that seen with *in vitro* testing, where there was non-linear release characteristics observed in the dissolution tests performed (Figure 4.8.9). In the *in vivo* studies, when results from the test pockets in dogs 1 and 2 were averaged, the delivery system released an apparently constant concentration of tetracycline, independent of time, from days 14 to 42, i.e. zero-order release. In order to confirm that there was no significant difference in concentrations between days 14 and 42 analysis of variance (ANOVA) was performed on the data. The results show that there was no difference between the average release over the 6 week period and the average release at days 14, 28 and 42 (Table 5.3.1) i.e. the release is time independent, verifying that the system shows apparent zero-order release.

Table 5.3.1 Analysis of variance for release of tetracycline base into periodontal pockets.

Source	*DF*	*SS*	*MS*	*F*	*P*
day	2	185	93	0.00	0.997
Error	31	1076806	34736		
Total	33	1076991			

This is similar to the release profile observed by Tonetti *et al.* (1990), using ethyl vinyl acetate fibers loaded with tetracycline HCl. However their average concentration was 1590 μg/ml over the 10-day period. It is difficult to draw an exact comparison with concentrations achieved in this *in vivo* study as their method of sampling was different, with gingival fluid samples being taken. However it is clear that the concentrations achieved using the tetracycline fiber were higher than with the product currently being tested. There is little significance in these high concentrations as concentrations achieved need only be higher than the minimum inhibitory concentration (MIC) of periodontopathic bacteria. The high concentrations achieved with the tetracycline fiber are a result of the nature of the product. The *in vitro* release rate of the fiber is estimated to be 35 μg/cm/hr for the first 2 hr and 2 μg/cm/hr thereafter, with the result that the concentration achieved in the pocket is dependent on the amount of fiber placed in the pocket and also the time sampling occurs at. Other tetracycline containing formulations evaluated *in vivo* for the treatment of periodontitis did not show zero order release. A cross-linked collagen film containing tetracycline showed rapidly decreasing concentrations from days 1 to 4 with concentrations in the region of 8 μg/ml being seen at day 10 (Minabe *et al.*, 1989). A novel bioerodible gel containing tetracycline HCl showed mean levels exceeding 100 μg/ml over 6 days (Needleman *et al.*, 1998), however from day 5 onward there was a rapid decrease in concentration over time.

It is also interesting to note that the tetracycline fiber produced a burst release (35 μg/cm/hr) during *in vitro* studies but that this was not observed with *in vivo* studies (Tonetti et al., 1990). The loss of the initial burst release with *in vivo* studies was

explained due to initial high release rates being absorbed by binding sites within the pocket due to the high binding capacity of tetracycline. Tetracycline solutions exhibited good ability to bind to tissues in the periodontal environment with retention half-lives of 4.4 hr and 12.2 hr (1% and 10% solutions), 250 and 700 times longer than the half-life of 0.017 hr expected for a substance that has a poor binding ability (Tonetti *et al.*, 1990). A burst effect was also seen with the formulation currently being tested during *in vitro* studies, however as samples were not taken in the first 24 hr of this study it is not known whether this burst release was observed *in vivo*.

When the average release of tetracycline in the test pockets was examined in the two dogs separately, an inverse relationship was observed (Figure 5.3.2). Dog 1 showed an increase in tetracycline levels from day 14 to day 28 with a subsequent decrease from day 28 to day 42. Dog 2 showed a decrease from day 14 to day 28 followed by an increase in tetracycline levels from day 28 to 42. It is also clear that the changes in concentration are far larger on a point-to-point basis in dog 1 than in dog 2. When examined using a paired t-test (95% confidence interval, $p<0.05$) the differences were not found to be statistically significant, showing that the release profile in the two dogs is not significantly different.

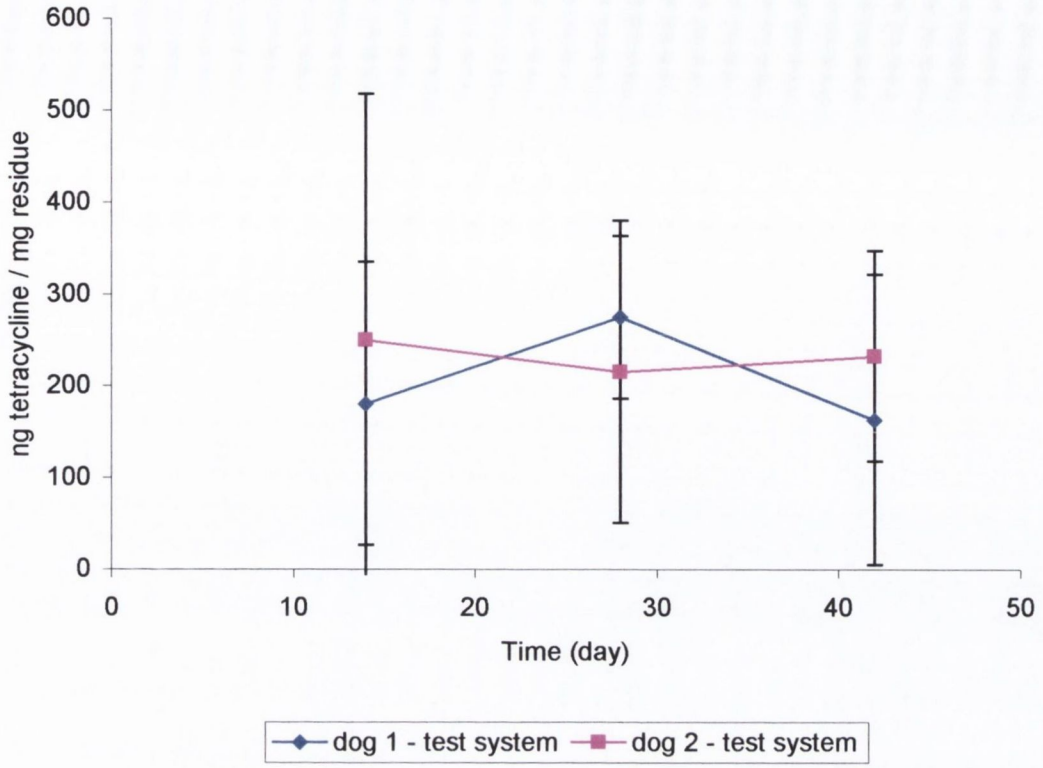


Figure 5.3.2 Average tetracycline base concentrations ng/mg of residue in test pockets of dog 1 and dog 2.

5.3.2 Average release from control pockets

The control pockets at all days showed detectable levels of tetracycline present, at day 14, 66.79ng/mg (6.68 μg/100 mg), day 28, 27.82 ng/mg (2.78 μg/100 mg) and at day 42, 57.86 ng/mg (5.79 μg/100 mg). However these levels are in all cases at least three orders of magnitude smaller than the test pockets and presumably arose by cross-diffusion from drug treated pockets. The presence of antibiotic in control pockets also occurred in a study performed by Needleman *et al.* (1998), where concentrations of up to 25% of the test pockets were observed, depending on the position of the control pockets in relation to the test pockets.

It is evident that there is a certain amount of crossover from the test pockets to control pockets and this can be considered a disadvantage to split-mouth trials. However the alternative is a whole mouth trial where either all test or control

samples are placed in one dog. The problem with that method is there is no way of ensuring that the oral environment of the different dogs is uniform, with the consequence that results can be biased and as a result difficult to interpret.

5.3.3 Release profiles in individual test pockets

The standard deviations observed in both Figure 5.3.1and Figure 5.3.2 are large and when concentrations of tetracycline base in individual pockets (Figure 5.3.3) are examined the reason for this is clear. Variations in concentrations of tetracycline in individual pockets are considerable with the test pockets in each dog not showing a consistent release pattern over time. In 5 of the 12 pockets (3 in dog 1, 2 in dog 2) one type of release pattern was observed, with an increase in tetracycline concentration from day 14 to 28 followed by a subsequent decrease from day 28 to 42. Two pockets in dog 1 showed a continual decrease in tetracycline concentrations over time. Three pockets (1 in dog 1 and 2 in dog 2) showed a continual increase in tetracycline concentration over time, while the final 2 pockets in dog 2 showed a sharp decrease from day 14 to 28 followed by a rapid increase in tetracycline concentrations from day 28 to 42. Increases over time could be due to tetracycline being released from binding sites within the pocket, where it can be held for long periods of time due to the binding capacity of the antibiotic.

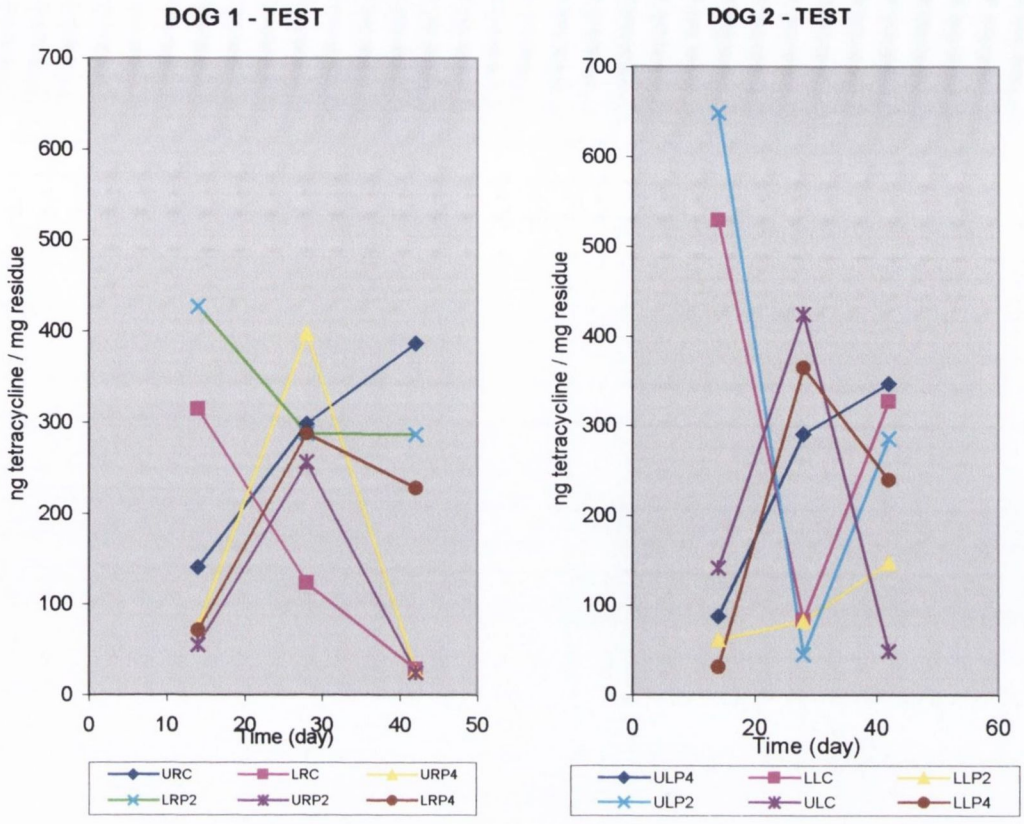
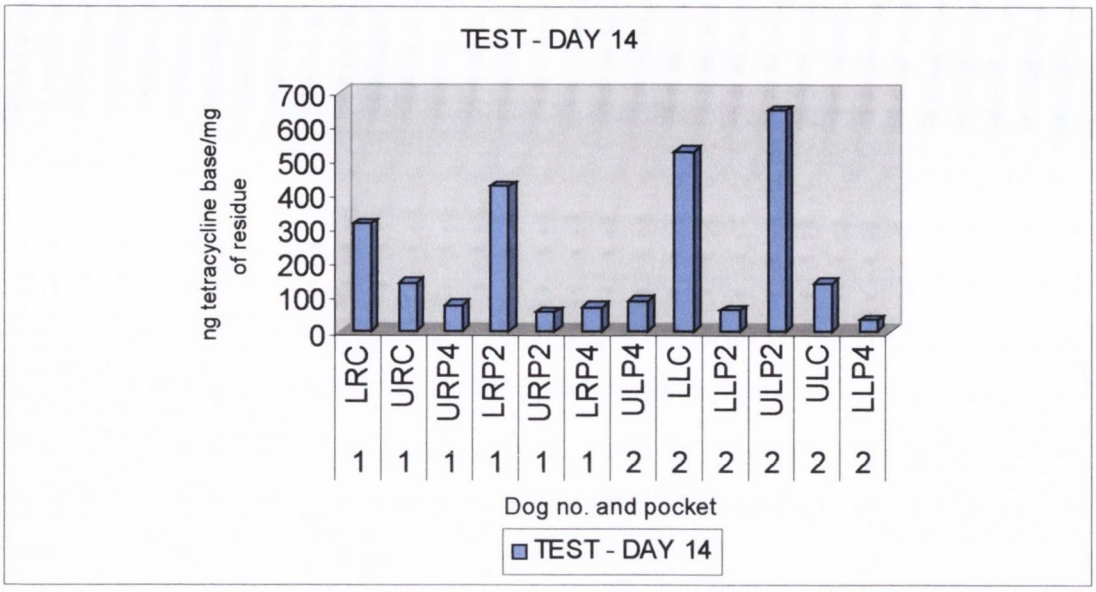


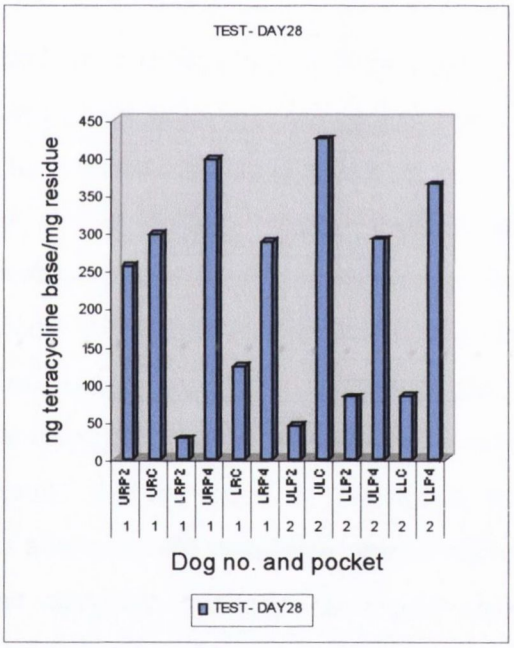
Figure 5.3.3 Level of tetracycline base over time in individual pockets

The level of tetracycline base in individual pockets at each time point is shown in Figure 5.3.4. At day 14 there was an average of 215.5 ng/mg residue of tetracycline contained in the test pockets. However this was not evenly distributed among the pockets. Four of the pockets showed significantly higher concentrations of tetracycline than the other pockets (2 in dog 1 and 2 in dog 2). At day 28, there were 2 distinct groupings in terms of levels of tetracycline present in the pockets. Seven pockets showed levels between 255 and 425 ng/mg tetracycline (4 in dog 1 and 3 in dog 2), while the other five pockets showed much lower levels of between 25 and 125 ng/mg. At day 42, seven of the pockets (3 in dog 1 and 4 in dog 2) showed concentrations between 225 ng/mg and 390 ng/mg, one pocket had an intermediate concentration of 147 ng/mg, while the other four pockets had values between 25 and 50 ng/mg. While these results point to a wide variation in levels present in pockets, it is clear that there is no bias between the two dogs with both showing similar ranges of levels and similar numbers of pockets within these level ranges.

5.3.4 Retention of the product

The residue studies clearly indicate that the retention of the product in the pockets was adequate. There was an almost constant release of tetracycline into the pockets for 6 weeks, which was only possible if the product had been retained for that time period. All products showed tetracycline base present for the 6-week period, except for one pocket at day 42. Retention of product in the pocket in many cases has proved to be a challenge with other drug delivery systems. A study of a bioerodible injectable system showed only 60% of samples positive for tetracycline (Schwach-Abdellaoui *et al.*, 2001), while gel systems were lost rapidly due to dilution of the system. Most studies published do not give details of how many samples were lost due to poor retention, so it is difficult to identify the extent of this problem. However according to anecdotal evidence, there is significant loss associated with, in particular, the *Periochip*™. This gelatin matrix is inserted into the pocket and has no retentive dressing applied and as a result can fall out quite easily.





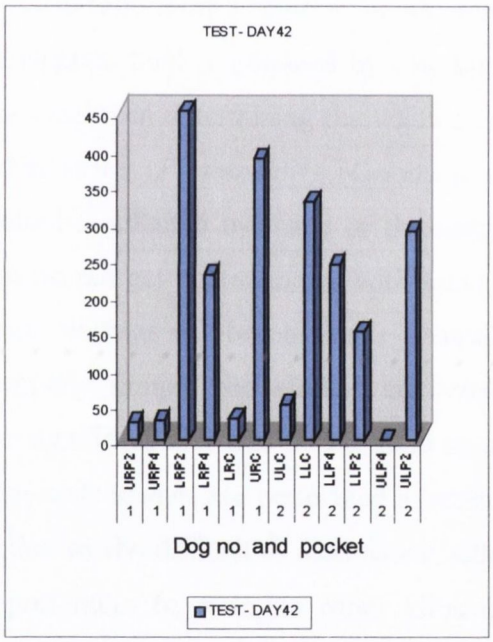


Figure 5.3.4 Tetracycline levels in individual pockets at each time point sampled.

5.3.5 Sampling method

The problems observed with variation in levels and release patterns within the pockets could be due to issues surrounding the sampling method used. The sampling method consisted of two sterile orthodontic paper points being inserted

into the base of the pocket for 30 s, then being removed and placed in a glass vial. There are two concerns with this method. Firstly the amount of residue taken varies greatly from pocket to pocket (Appendix 8) and there is no way to standardise this to ensure that the same amounts are taken from each pocket. The second problem concerns the non-specific nature of the sample obtained. The residue obtained as sample could consist of three different components, gingival crevicular fluid, blood and product residue. Depending on the amount of each component in the residue obtained, the levels of tetracycline base detected will differ significantly. If there is mainly product residue, tetracycline levels could be high whereas with samples containing mainly blood and gingival crevicular fluid, levels of tetracycline base would be expected to be lower.

The more common method for sampling concentrations of antibiotic in gingival pockets is using gingival fluid volume. The gingival fluid is obtained by inserting filter paper strips into the pocket until 80% wet and then determining the volume of fluid obtained by using a gingival crevicular fluid meter (*Periotron™),* (Goodson *et al.*, 1991a, Killoy, 1998). However this method is affected by many of the same problems, it is difficult to ensure that the strips do not get contaminated with saliva, blood and plaque residue, and if they do, biased readings will be obtained. Overall the gingival pocket is a difficult site to properly sample and studies involving sampling the pocket will always be subject to significant amounts of variation as a result. It is important for this reason, that large-scale studies are performed in order to allow for the variability, which will occur due to the difficulties associated with the sampling method. It would also be preferable to examine other clinical parameters e.g. bleeding on probing, gain in clinical attachment, probing depths, in order to ensure meaningful results are obtained.

5.4 *In vivo* microbiological counts

5.4.1 Test pocket counts *vs.* control pocket counts

Microbiological testing to determine total numbers of aerobic and anaerobic colony forming units (CFU's) was performed at days 0, 14, 28, 42 and 56. For a complete

list of counts obtained refer to Appendix 9. Table 5.4.1 and Table 5.4.2 show the numbers of CFU's in test pockets in dogs 1 and 2, along with the overall average during the 56 day test period. Table 5.4.3 and Table 5.4.4 provide the same information for the control pockets. This data is depicted graphically in Figure 5.4.1 and Figure 5.4.2.

Table 5.4.1 Average aerobic counts (CFU's) for test pockets (6) in each dog.

	Dog 1	***Dog 2***	***Average***	***Standard deviation***	***% standard deviation***
Day 0	56	87	71.5	20.50	28.28
Day 14	280	133	206.5	40.31	19.52
Day 28	218	205	211.5	9.19	4.35
Day 42	220	570	395	247	62.53
Day 56	172	298	235	89.8	38.21

Table 5.4.2 Average anaerobic counts (CFU's) for test pockets (6) in each dog.

	Dog 1	***Dog 2***	***Average***	***Standard deviation***	***% standard deviation***
Day 0	56	87	71.5	20.5	28.28
Day 14	150	35	92.5	81.32	87.91
Day 28	90	157	123.5	47.38	38.36
Day 42	130	630	380	353.55	93.04
Day 56	298	86	197	57.98	76.10

Table 5.4.3 Average aerobic counts (CFU's) for control pockets (6) in each dog.

	Dog 1	*Dog 2*	*Average*	*Standard deviation*	*% standard deviation*
Day 0	56	87	71.5	20.50	28.28
Day 14	715	790	752.5	53.03	7.05
Day 28	898	657	777.5	171.12	22.01
Day 42	730	640	685	63.63	9.29
Day 56	188	265	226.5	82.73	36.52

Table 5.4.4 Average anaerobic counts (CFU's) for control pockets (6) in each dog.

	Dog 1	*Dog 2*	*Average*	*Standard deviation*	*% standard deviation*
Day 0	56	87	71.5	20.5	28.28
Day 14	511	470	490.5	28.99	5.91
Day 28	670	318	494	248.90	50.38
Day 42	390	506	448	82.02	18.31
Day 56	213	295	254	199.4	78.50

Both control and test pockets showed similar low aerobic and anaerobic counts at day zero (Figure 5.4.1 and Figure 5.4.2). This is as would be expected, as there is no significant difference between the pockets at this time point, due to the fact that they had been surgically created just prior to having the test product delivered into them.

By day 14 there had been a 3 fold increase in the average number of aerobic organisms (Table 5.4.1) and a 1.3 fold increase in the average number of anaerobic organisms in the test pockets (Table 5.4.2). In the control pockets there had been a 10.7 fold increase in aerobic organisms (Table 5.4.3) and a 7 fold increase in anaerobic organisms (Table 5.4.4). The increase in the number of CFU's is to be

expected as the pocket has experienced a surgical trauma and opportunistic bacteria would take advantage of this situation (Renvert *et al.*, 1996). The reduction in total number of CFU's seen in the test pockets compared to the control pockets, is due to the antimicrobial activity of the locally liberated tetracycline base. This tetracycline base is being released from the halloysite system into the pocket over time. Tetracycline base is active against both aerobic and anaerobic microorganisms; hence it would be expected to cause a reduction in the numbers of microorganisms in the test pockets.

At day 28, there was no change in the number of aerobic CFU's present in the test pockets, while there was a 33% increase in the number of anaerobic CFU's (Figure 5.4.1). There was no change in the average number of aerobic or anaerobic CFU's in the control pockets, which were still several orders of magnitude greater than the test pockets (Figure 5.4.2).

At day 42, there was a 3 fold and a 1.9 fold increase of anaerobic and aerobic CFU's respectively in the test pockets (Figure 5.4.1). At day 42, the control pockets showed continuing high levels of both aerobic and anaerobic CFU's (Figure 5.4.2). Even after the increase in total CFU's in the test pockets at day 42, they were still 23% and 45% lower than the control pockets for anaerobic and aerobic counts respectively.

At day 56, both test and control pockets showed a drop in counts almost returning to the zero day baseline as healing of the pockets occurred (Tables 5.4.1 - 5.4.4).

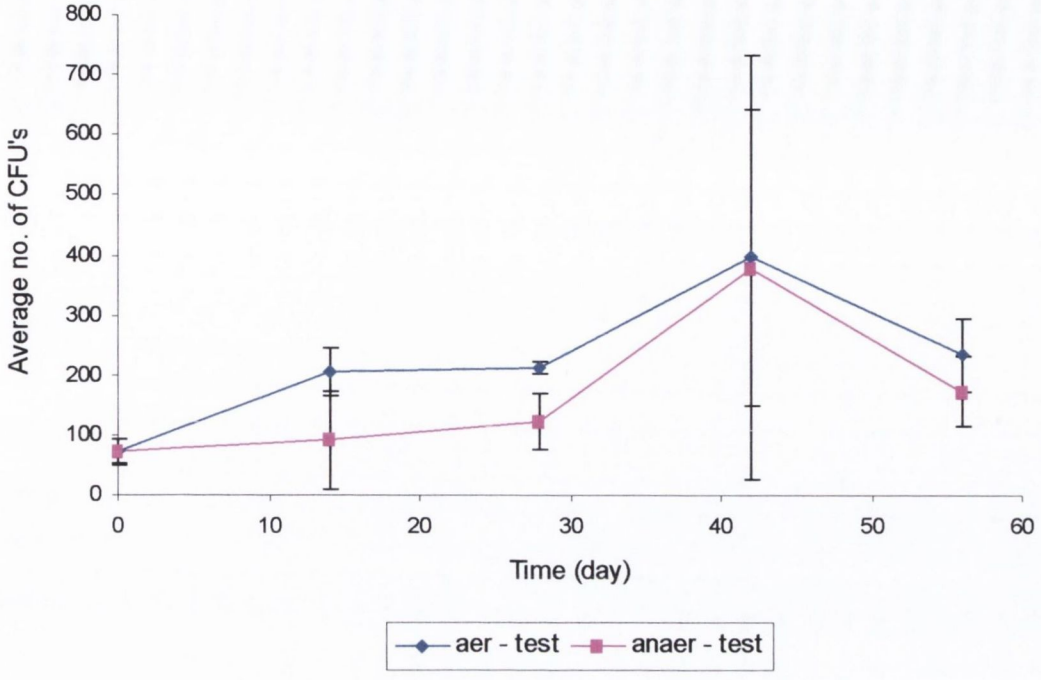


Figure 5.4.1 Average aerobic and anaerobic counts for test pockets (dogs 1 and 2).

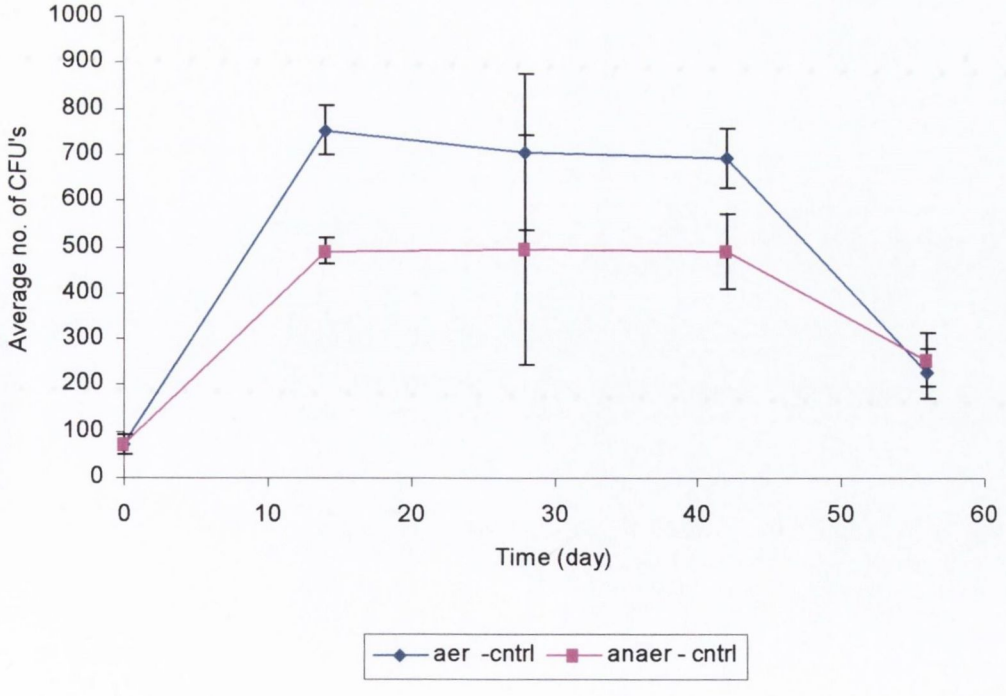


Figure 5.4.2 Average aerobic and anaerobic counts for control pockets (dogs 1 and 2).

When ANOVA was performed on the differences between numbers of colony forming units for both aerobic and anaerobic bacteria in test and control pockets the results were shown to be significant in both cases. For aerobic counts a p value of 0.004 and for anaerobic counts a p value of 0.015 was achieved using the data from days 14, 28 and 42. This finding shows that the tetracycline present in the pockets is having a significant positive effect in reducing microbial counts in the test pockets at day 14 and 28.

Figure 5.4.3 and Figure 5.4.4 highlight the significant differences in aerobic and anaerobic counts between the test and control pockets over time. The suppression that occurs in the test pockets over the first 28 days can be clearly seen with the subsequent return to baseline that follows. However the counts seen for the control pockets could be artificially low due to the results obtained in the residue studies. At all time points low levels of tetracycline were found in the control pockets as well as the test pockets due to crossover contamination. This would lead to suppression of counts of CFU's in the control pockets to a certain extent. As a result, values seen here would not necessarily represent the full magnitude of the difference between test and control pockets.

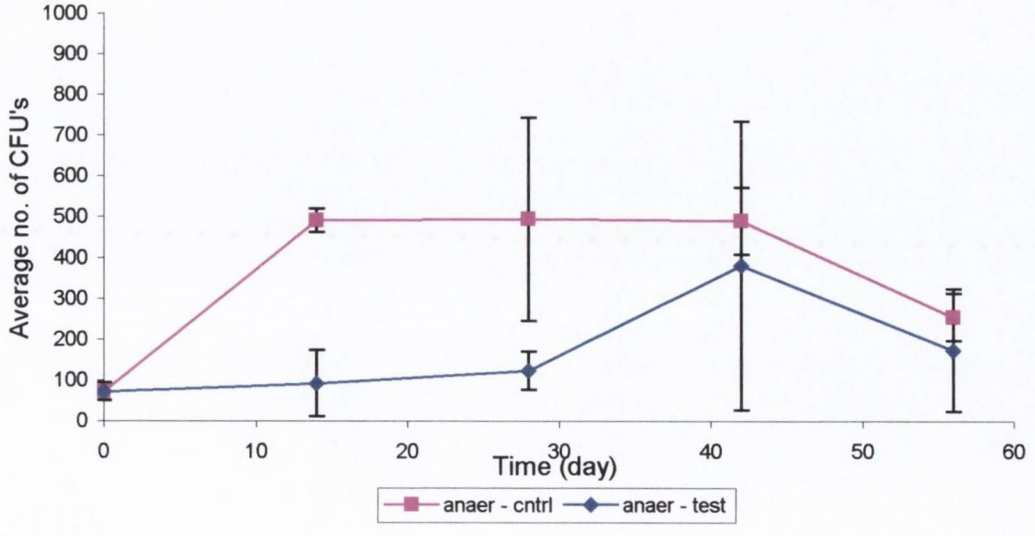


Figure 5.4.3 Average anaerobic counts for control and test pockets (dogs 1 and 2).

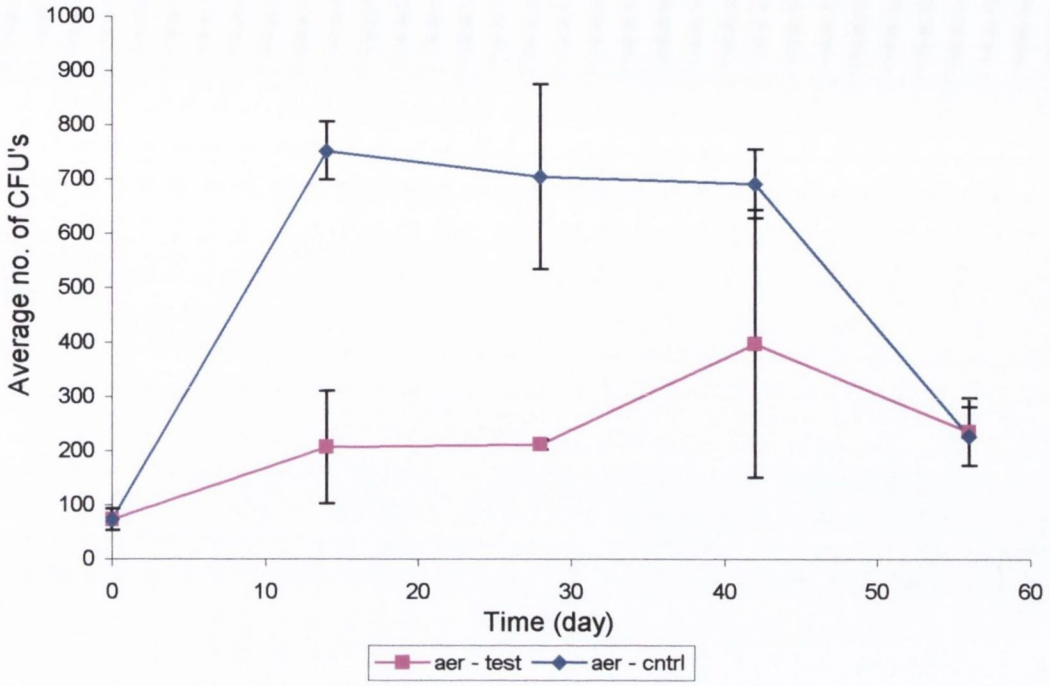


Figure 5.4.4 Average aerobic counts for test and control pockets (dogs 1 and 2).

5.4.2 Sample size considerations

The sharp increase in counts, seen at day 42 for both aerobic and anaerobic test pockets warrants further investigation. It is clear that the standard deviations in the test pockets for both aerobic and anaerobic counts at day 42 are much larger than at any other time point for the test pockets (Table 5.4.1). When aerobic and anaerobic counts (test pockets) for individual dogs were examined, it was observed that a sharp increase in both counts was observed in dog 2 at day 42. In dog 1 there was a 1.4 increase in anaerobic counts and a 1.1 increase in aerobic counts. In dog 2 these values are 4 fold and 2.8 fold increases respectively. When microbial counts for day 42 in dog 2 were examined more closely, it was found that there were two pockets that were showing extremely high counts, compared with all other pockets. If these two outliers were excluded from the data (Figure 5.4.5), it caused a visible difference to the data distribution that is seen when the two outliers are included (Figure 5.4.6). Figure 5.4.5 and 5.4.6 show the average concentration of tetracycline in the test pockets overlaid onto the average aerobic and anaerobic

counts in the test pockets at the different time points. At day 42 (including the two outliers - Figure 5.4.6) there is a large increase in aerobic and anaerobic counts even though there is little difference in the tetracycline levels present in the test pockets. If the two outliers are excluded from the data (Figure 5.4.5), a clear difference can be seen with aerobic and anaerobic counts being reduced to levels only slightly above day 28 levels. This result would correlate with the tetracycline levels detected. The average levels of tetracycline present in the pockets over the 6-week test period were almost constant and therefore it would be expected that bacterial counts in the pockets would remain almost constant also.

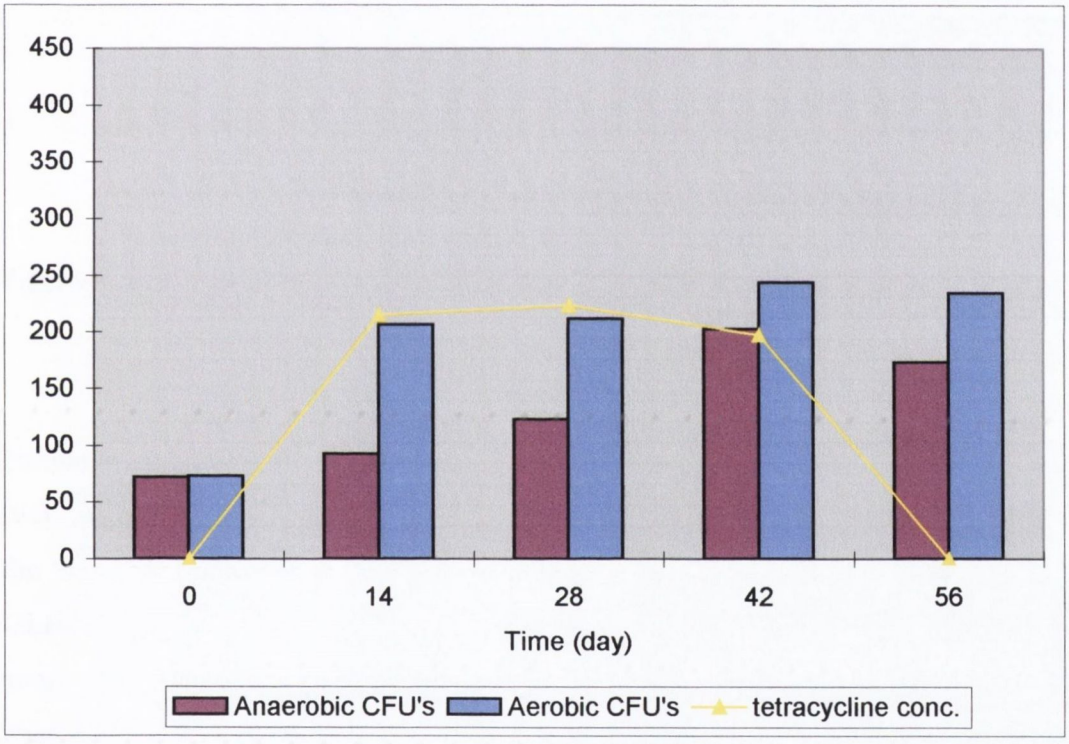


Figure 5.4.5 Comparison of aerobic and anaerobic counts and tetracycline levels (test) excluding the two outliers at day 42.

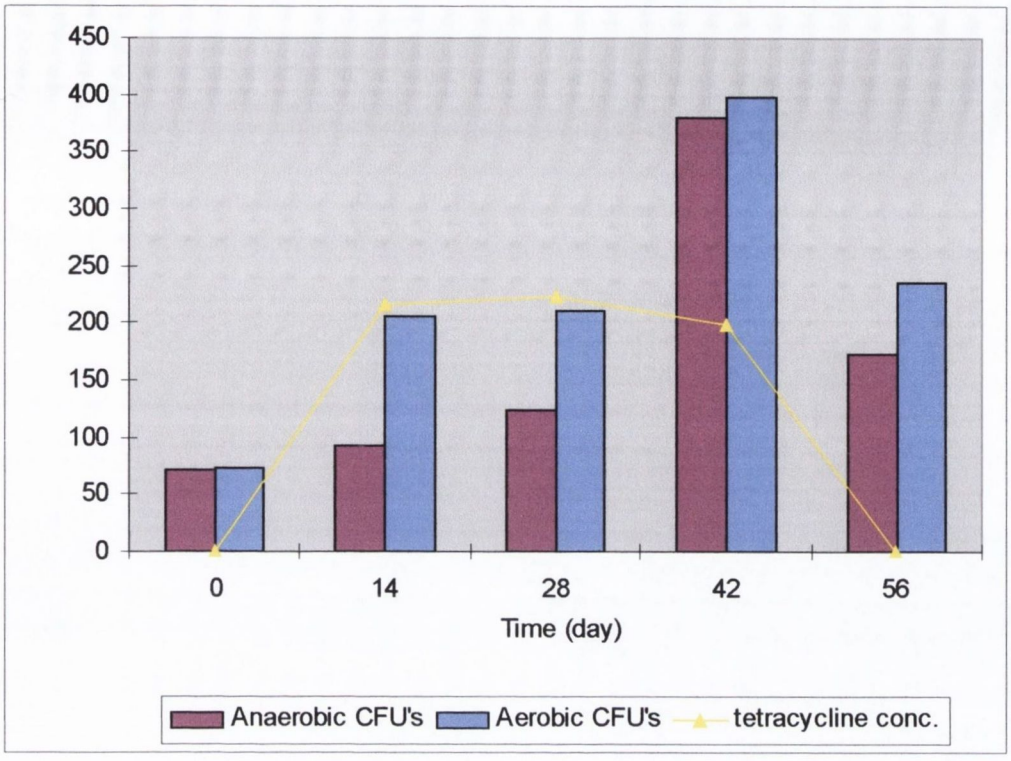


Figure 5.4.6 Comparison of aerobic and anaerobic counts and tetracycline levels (test) including the two outliers at day 42.

However the question still remains as to why these high counts have occurred. The two pockets affected were LLP4 and ULP4. When the counts were overlaid with the tetracycline levels in these pockets, it can be seen that at day 42 the test pocket ULP4 showed no tetracycline present, implying a loss of product in that pocket at some point between days 28 and 42 (Figure 5.4.7). This could account for the high microbial counts observed here. However this cannot apply to the other pocket, which shows adequate levels of tetracycline present. The only other possibility is that the sample was contaminated in some way either through incomplete sterilization of the vial, although this is unlikely as all the vials were sterilized together, during plating or contamination during the sampling procedure.

A problem with this study is emphasised with the skewed results seen at day 42. This was the small sample size used. The effect of using such a small study is clearly seen, where two anomalous results skewed the whole data set significantly. It would be preferable to have a much larger sample size in a study of this nature,

where excess variability is likely to be a problem. Large variability is inherent in animal studies, however it is likely to be even larger here due to the small sample sizes being obtained and also due to the ease with which samples could become contaminated. All other time points other than day 42 show similar results between dogs 1 and 2 for test pockets (Figure 5.4.7) and therefore there was no significant skewing of the data seen.

The other issue with this study was the lack of specificity with regard to the microbiology. Due to certain constraints, it was only possible to examine total colony forming units for aerobic and anaerobic bacteria. As a result it is not known which species and their numbers were present, or which of the microorganisms present were most affected by the treatment delivered. There is no knowledge if there were any resistant species present or if any showed increased resistance over the time period involved.

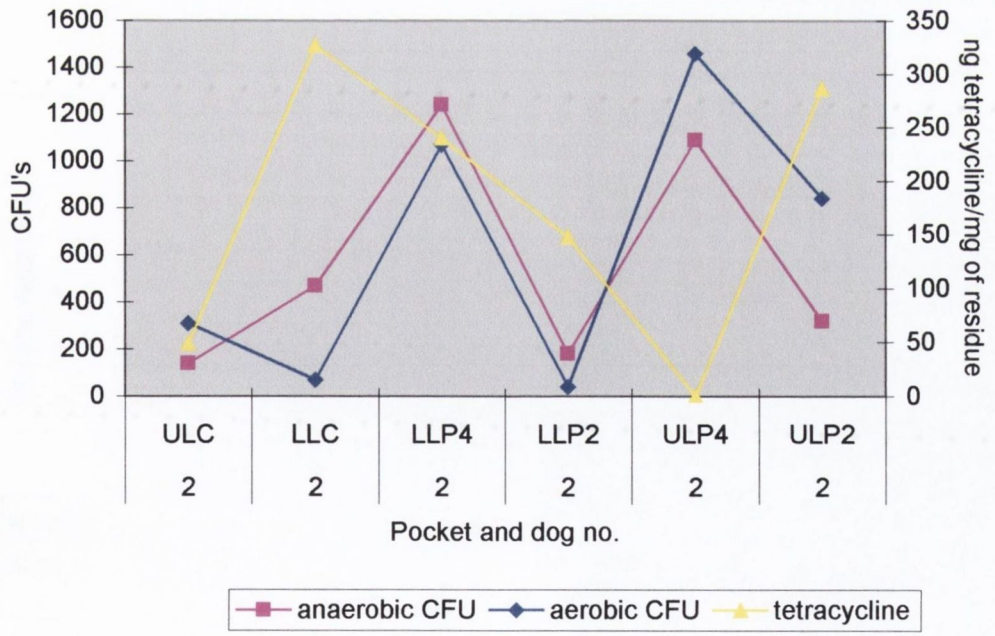


Figure 5.4.7 Correlation between colony forming units and tetracycline levels at day 42.

5.5 Probing pocket depth results

Reduction in probing pocket depth (PPD) is a parameter used to determine clinical improvement in the treatment of periodontitis. Probing depths of pockets were taken also at all sampling time points. Individual results for all samplings are given in Appendix 10.

There is a continuous reduction in PPD from day 14 to day 56 in the test pockets. The control pockets also show some change over time, however it is smaller in extent. The largest decrease in PPD in the test pockets was seen between days 14 and 28, where there was a decrease of 0.85 mm. This improvement was maintained and even slightly improved over the following 2 weeks. The PPD in this particular study is not as important as it would be in a study performed on patients suffering from periodontitis. The pockets in this case were surgically created, and therefore will heal and reduce in depth in a manner different to pockets that develop as a result of periodontitis. It is difficult as a result to determine the significance of the PPD data determined in this study.

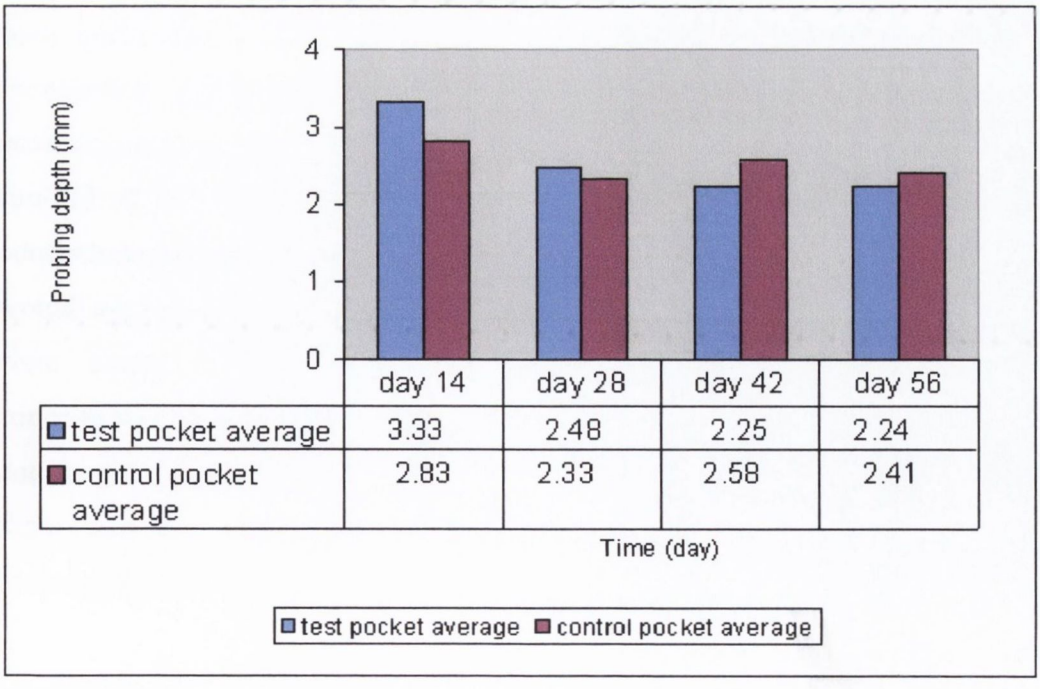


Figure 5.5.1 Probing depths of test and control pockets.

5.6 Conclusions

The formulation tested had a number of properties that are considered essential for a product to be used for the localized treatment of periodontitis. It offered ease of application due to the fact that it was syringeable at room temperature. It gave controlled release for a period of up to 6 weeks, through the use of halloysite to control the release of tetracycline base. Good retention was provided by the gelling ability of the thermoresponsive *poloxamer 407* at body temperature, coupled with the addition of octyl CA, a strong tissue adhesive.

The final formulation developed was tested *in vivo* in dogs to determine its potential clinical activity. Due to the unlicensed nature of the novel excipient halloysite this testing was performed in a dog model, in which pockets were surgically created. Due to the fact that surgery was performed on the oral mucosa of the dogs, it was difficult to determine whether the product caused any local irritation to the pockets. The results obtained showed that the release of the tetracycline over a 6-week period was roughly zero order, with an average release of 21.24 μg/100 mg of residue. Tetracycline has been shown to be active against both gram-positive and gram-negative bacteria. In terms of periodontopathic bacteria, a number of studies have been performed to determine the activity of tetracycline. *In vitro* activity of tetracycline against clinical isolates of *P. gingivalis, P. intermedia* and *A. actinomycetecomitans* gave 90% MIC (minimum inhibitory concentrations) values (μg/ml) of 0.5, 0.25 and 4 respectively, with 90% MBC (minimum bactericidal concentrations) values of 16, 0.5 and 64 μg/ml (Miyake *et al.*, 1995). Therefore it would appear that the concentrations of tetracycline achieved in the test pockets were bacteriostatic at the very least and could potentially have reached concentrations that were bactericidal. However it is difficult to fully ascertain this due to problems associated with the sampling method. The results do definitively show that the product had sufficient retention to remain in the pocket for a prolonged period of time.

Microbiological studies performed further verified the positive impact that the antibiotic had on the microbiological activity within the pocket. It is clear that the system tested is capable of suppressing microbiological activity (both aerobic and

Chapter 6

Formulation of a product for the symptomatic treatment of xerostomia

6.1 Theory of formulation

6.1.1 Introduction

Saliva is produced by three pairs of major salivary glands, the parotid, sublingual and submandibular glands. The parotid glands, which lie just below and in front of the ears, are the largest. The sublingual glands are found under the tongue, while the submandibular glands are located close to the sides of the jaw. The oral cavity is also lined with a mucous membrane that contains numerous minor salivary glands. When salivary production is occurring the parotid glands are the primary source of saliva, however when there is no stimulus, the sublingual glands are responsible primarily for salivary production, while the submandibular glands account only for 2.5% of total saliva production (Garg and Malo, 1997). Saliva has numerous functions, which have been discussed in Section 1.7, and is extremely complex both in its composition and function, and in the interplay that occurs between many of its components. The complexity of saliva along with its numerous interrelated functions and the sophistication of its release system in the oral cavity make it a difficult substance to mimic artificially.

Saliva plays numerous important functions in the oral cavity e.g. lubrication, coating, antibacterial, and the loss of salivary function will result in a host of problems. Xerostomia sufferers typically are more prone to gingivitis, periodontitis, *Candida* infections and increased incidence of dental caries (Plankhurst *et al.*, 1996, Little, 1986, Soto–Rojas *et al.*, 1998, Guchelaar *et al.*, 1997), as well as suffering the discomfort of a dry mouth, which can lead to difficulty with mastication and swallowing (Hamlet *et al.*, 1997). In order to try and minimize the damage to the oral cavity due to the loss of saliva, a saliva substitute should try to mimic the functions of saliva as closely as possible. It was considered that an ideal saliva substitute should offer lubrication of the oral cavity for a prolonged period of time and help provide protection against the many oral conditions that xerostomia patients are prone to suffer from, however the product

must also be acceptable in terms of consistency and taste so that patients will be encouraged to use it on a long term basis.

6.1.2 Mineral function

Increased incidence of caries is a significant problem associated with xerostomia, as the excretion of minerals in saliva is lost and the pH of the oral cavity is reduced leading to demineralization of the teeth. The functions of the ions present in saliva are to contribute to the osmolarity of saliva and also to its buffering capacity. Calcium and phosphate in saliva are present both free in solution and in soluble complexes with carbonate, lactate and also as calcium phosphate. Calcium is also found bound to proteins. Despite these non-ionic complexes, the levels of calcium and phosphate are such that saliva is supersaturated with respect to hydroxyapatite at normal intra-oral pH. The reaction for the dissolution of hydroxyapatite is described by:

$$Ca_{10}(PO_4)_6(OH)_2 \rightleftharpoons 10Ca^{+2} + 6PO_4^{3-} + 2OH^-$$

Equation 6.1.1 Reaction for dissolution of hydroxyapatite.

The role of calcium and phosphate in saliva is as follows; if pH decreases, the concentration of ions needed for saturation increases and at a pH of approximately 5.6, tissue starts to dissolve to maintain saturation. The lower the pH the faster the demineralization. When the pH falls (i.e. the H^+ concentration increases) the PO_4^{3-} are converted to HPO_4^{2-} or $H_2PO_4^-$ (i.e. they take up protons) and the OH^- ions are neutralized to form water. This causes the saliva to be no longer supersaturated and drives the reaction to the right (dissolution), thus slowing down or reversing the fall in pH which in turn drives the reaction to the left (remineralization) as the degree of supersaturation increases. This cyclical change that occurs in pH in the oral cavity is known as the 'Stephan curve'. When carbohydrate rich or sugary foods are taken this

stimulates salivary flow, which in turn increases the buffering capacity of saliva and also helps wash out remaining acids and sugars and this contributes to the pH rising phase of the 'Stephan curve'. During this recovery phase plaque gradually becomes supersaturated with hydroxyapatite (HAP) and mineral precipitation may occur.

The buffering capacity of saliva is directly related to its flow, with the difference in the buffering capacities of resting and stimulated saliva related to the sodium and bicarbonate concentrations. Both sodium and potassium were included in the formulation, as potassium is the predominant cation in the buffer systems of resting saliva but sodium predominates in stimulated saliva. It is clear from the above, that the presence of the salts in the formulation are important in helping prevent further caries formation in xerostomic patients. Their oral cavities are lacking in essential minerals naturally and there will be no natural buffering capacity in the oral cavity when the pH falls.

Phosphate was chosen as the major buffer as it plays a dual role in the prevention of caries, acting both as a buffer and as a component of hydroxyapatite (HAP). While there is a greater amount of bicarbonate than phosphate in human whole saliva, this would appear to be related to the fact that sodium and bicarbonate are present in the extracellular component of blood. They are available in forms that pass readily into and are released by the glands contributing to the formation of human saliva. By comparison phosphate, proteins and potassium are mainly intracellular components. Their presence in saliva represents materials stored in the secretory cells, which have been converted into a suitable form and subsequently discharged (Driezen *et al.*, 1953). Therefore supplementing the bicarbonate with the phosphate would not appear to be detrimental to the remineralization cycle.

A number of minerals in the form of salts were therefore included in the formulation to try to reduce the incidence of caries in the xerostomic patient by ensuring that the minerals necessary for the remineralization of the teeth were available in the oral cavity.

The salts included in the formulation are found in the product *Luborant*™ in similar quantities to that used in the new formulation. These quantities are comparable to those levels of minerals found in natural whole saliva (Table 6.1.1). This underlies the belief that a saliva substitute should mimic the properties of natural saliva as closely as possible.

Table 6.1.1 Mineral and buffer composition of whole saliva (Edgar, 1992)

Mineral	***Content in whole saliva (mg/100ml)***
Sodium	0 - 80
Potassium	60 - 100
Calcium	2 - 11
Phosphorous	6 - 71
Bicarbonate	0 - 40
Fluoride	0.01 - 0.04
Chloride	50 - 100

6.1.3 pH of saliva

The pH of the product is 6.75 ± 0.05. The pH of the oral cavity varies from 5.3 where there is a very low flow rate to 7.8 at high parotid flow rates. This change in relation to flow rate is due to the increased excretion of bicarbonate as salivary flow rate increases. However xerostomic patient's oral cavity pH tends to be low due to the loss of the buffering capacity of saliva. A pH of 6.75 was chosen for our formulation as it is close to neutral and therefore should have no detrimental effects on the oral cavity. It should also be noted that a lower pH might have an irritant effect on the oral mucosa.

6.1.4 Fluoride

Fluoride is an important mineral in caries prevention and therefore is of particular relevance to xerostomic patients. Fluoride is not found in the diet to any large extent and only small quantities are secreted in the saliva, with unstimulated saliva containing a higher concentration than stimulated saliva. With xerostomic patients, depending on the cause of the xerostomia, saliva flow can be severely restricted or non-existent and caries prevalence is high as a result of the shift in the pH profile, which results due to the lack of saliva production. Therefore fluoride supplementation is required to further improve the patients' chance of maintaining good oral health.

The role of fluoride in caries prevention is complex and still not fully understood, with a number of theories being put forward as to how it works. The mineral phase of enamel is known as hydroxyapatite (HAP), this has an unusual property in that different ions can occupy positions in the crystal lattice without causing major changes in form or dimension. Pure HAP interacts with concentrations of fluoride up to 100 ppm by exchanging a hydroxyl for a fluoride ion to form fluorohydroxyapatite, which can go to completion to form afluoroapatite. With concentrations in excess of 100 ppm calcium fluoride is formed. Calcium fluoride gradually dissolves acting as a reservoir of fluoride that can enter plaque and diffuse into enamel. The retention of calcium fluoride on the teeth is longer than would be expected, considering its solubility. This is probably due to it being coated with salivary proteins, which decrease its solubility. Its action in caries prevention is the result of the combined effect of several different actions, with three main hypotheses being proposed to account for the anti-caries effect:

1. Fluoride affects the morphology of the teeth, with the occlusal surfaces becoming more rounded, making them more self cleansing. However this effect is generally considered too small to be of practical importance.

2. Fluoride makes apatite less soluble and therefore more resistant to the acids formed by plaque bacteria, while also favouring remineralization. There has been contradictory evidence in this area, with *in vivo* studies showing that fluoride makes apatite less soluble while *in vitro* studies suggest that the concentrations of fluoride on the outer enamel is too low to exert this effect. There is also a suggestion that the effect of fluoride on solubility is related to carbonate. Carbonate is the mineral lost in the first stages of caries. When HAP is formed *in vivo* in a low fluoride area, carbonate ions tend to attach to the growing crystals and block the take up of phosphate, which makes these crystals more soluble. A higher concentration of fluoride reverses this tendency by accelerating the growth of the crystals and decreases their carbonate content.

3. Fluoride is an enzyme inhibitor and might be expected to reduce bacterial acid production. Another mode of action relates to its weak acid character, which acts to reduce the acid tolerance of bacteria.

(Murray *et al.*, 1991)

Overall the anticaries action of fluoride appears complex, with effects on both the mineral phase and bacteria. However it has been shown to be effective in caries prevention and therefore it is an important mineral to include in any xerostomia formulation as caries is a significant problem for xerostomia patients.

6.1.5 Flavour

Xerostomia is often a long-term condition and in many cases a life long problem for patients. Consequently the flavour of the formulation is extremely important, as the product needs to be used on a continual basis. Patients will not use the product if they do not find the taste acceptable and will lose the associated potential benefits that come with using it. It is also preferable that the product gives a 'fresh' feeling to the mouth

when used, as patients with xerostomia often develop a bad taste in their mouth. The development of an acceptable flavour in the product was difficult due to the presence of triclosan and sodium lauryl sulphate, which have unacceptable tastes. However it was felt that their inclusion in the formulation was necessary and therefore considerable effort was applied to masking their taste. Triclosan has a phenolic flavour, which if correctly masked confers a fresh mouth feeling, however if concentrations are too high it gives an unacceptable bitter aftertaste.

Xylitol and sorbitol were chosen as the sweetening agents because they are non-cariogenic, while a lemon flavour was selected due to its citrus, fresh taste. The lemon flavour was a spray-dried mixture of natural flavouring substances with maltodextrin, gum arabic, citric acid and L-ascorbic acid. It has an odour and taste typical of fresh lemon peels (Dragoco product specification). It is used in the manufacture of *Rubex*™ lemon (Ricesteele), a vitamin C effervescent and chewable tablet. Xylitol was the major sweetener used as it is fermented to acid more slowly than sorbitol, and therefore it does not depress plaque pH. This is a favorable action as low pH leads to demineralization, resulting in caries formation, which is already a significant problem in xerostomic patients. However it was found that xylitol alone was not capable of sweetening the product sufficiently, so sorbitol was also used. High concentrations of sorbitol alone left an aftertaste so a combination of the two with xylitol as the predominant sweetener was developed. Xylitol has been shown to have a tolerance of 200 g as oral doses daily and has been approved for use in foods in the United Kingdom. Sorbitol has an acceptable daily in-take, according to the WHO of 150 mg/kg of body weight (Handbook of Pharmaceutical Excipients).

6.1.6 Anti-microbial agents (e.g. Triclosan, sodium lauryl sulphate, zinc)

Xerostomic patients are prone to a number of conditions related to a shift in the microbial flora of the oral cavity, which occurs as a result of the loss of salivary function

e.g. gingivitis, *Candida*, ulceration of the mouth and increased incidence of dental caries (Plankhurst *et al.*, 1996, Little, 1986, Soto–Rojas *et al.*, 1998, Guchelaar *et al.*, 1997). Saliva has a number of antimicrobial agents present as a defense mechanism against invasion by pathogenic bacteria e.g. lysozymes, histatins, peroxidase and lactoferrin (Whelton, 1996). Another effective antibacterial mechanism of saliva is its washing action i.e. swallowing results in clearance of large numbers of bacteria. A reduction in saliva production causes a rapid increase in the population and type of microorganisms present in the mouth (Brown *et al.*, 1975). As a result of this an antimicrobial agent was considered beneficial in order to try and help reduce the problems associated with the loss of these natural antimicrobial functions of saliva, with an agent effective in the oral cavity being the material of choice.

Triclosan (2,4.4 prime-trichloro-2 prime-hydroxydiphenyl ether) was the agent chosen, as it is already present in a number of proprietary products e.g. *Colgate*™ toothpaste, *Sensodyne*™ mouthwash (Rosling *et al.*, 1997). Triclosan is a non-ionic, broad spectrum, antimicrobial agent, which is practically insoluble in water, moderately soluble in alkaline solutions, and readily soluble in most non-polar organic solvents (Kumar specifications). The safety of triclosan has been established, with it showing no acute oral toxicity, carcinogenic, mutagenic or teratogenic properties (Bhargava and Leonar, 1996). The sample supplied for use in this formulation was a special grade known as Triclosan 5000 (Kumar Organic Products), which is intended for use in oral products. Triclosan has been shown to be an effective antimicrobial agent for products used in the oral cavity with broad-spectrum activity against both gram-positive and gram-negative organisms, with the agent affecting gram-negative anaerobes to a greater extent than gram-positive groups (Saunders *et al.*, 2000). In a 4-day plaque regrowth test, it was shown that triclosan and sodium lauryl sulphate have plaque inhibitory properties (Jenkins *et al.*, 1991). It has also been observed that the use of triclosan in conjunction with sodium lauryl sulphate gives greater plaque inhibiting properties than triclosan used in conjunction with *Tween 80* or as a alkaline solution; this is possibly due to the strongly charged micelles formed by the use of sodium lauryl sulphate (Waaler *et al.*,

1993). An added advantage to the use of triclosan is that it has been shown to have a direct anti-inflammatory effect on the gingival tissues. This is helpful as inflamed gums, which occur due to lack of moisture is a common complaint of xerostomic patients. Gaffar *et al.* (1995) found that triclosan inhibited several important mediators of gingival inflammation. This study also suggested that the necessary triclosan concentrations could probably be achieved in local tissues, such as the gingiva, from topical applications.

In order to incorporate it into the formulation, sodium lauryl sulphate was chosen as a solubilizing agent at a concentration of 1%. This was chosen as the solubilizing agent of choice as it has been shown to have a synergistic action on the antimicrobial effect of triclosan. Sodium lauryl sulphate is already used in a number of pharmaceutical preparations such as a wetting agent in dentrifices, tablet lubricant and anionic emulsifier. Its safety is well established with an LD_{50} value of 1.0-2.7 g/kg in rats for acute oral toxicity. It has been shown to be irritating to the skin only in high concentrations e.g. application of 10% sodium lauryl sulphate (Handbook of Pharmaceutical Excipients).

Zinc has been shown to exert a modest anti-plaque effect in simple solutions (Harrap *et al.*, 1983), but when combined with triclosan the anti-plaque effect is greatly enhanced, to the extent that a gingival health benefit can be achieved (Svatun *et al.*, 1987). In a study of the maintenance of gingival health using a zinc and triclosan dentifrice, gingival health and plaque levels were similar after 6 months to the baseline, following scaling and oral hygiene instruction. The placebo group failed to maintain the same standard of oral hygiene. Zinc also performs possibly another function in the formulation. A constant complaint of xerostomia is the loss of taste sensation that patients suffer from. When zinc was included in a formulation in an uncontrolled study, there was some evidence that zinc therapy may be useful in ameliorating taste impairment (Mossman and Henkin, 1978).

6.1.7 Lubrication

The lubricating ability of saliva is provided by a slimy viscoelastic coat termed mucus, consisting predominately of salivary glycoproteins, proteins and lipids that coats the oral cavity. The unique physiochemical and rheological properties of the mucus coat are contributed largely by the mucin glycoproteins. Their functions include hydration and lubrication of the oral structure, and facilitation of the swallowing movement by producing a slippery bolus. This is one of the most important effects that needs to be imitated in an effective saliva substitute, as it is the loss of the lubricating effect of saliva that causes many of the physical difficulties encountered by xerostomic patients e.g. ease of swallowing and speaking.

The final product developed in this project contained sunflower oil as a lubricant to try and provide a lubricating effect in some way similar to that of normal saliva. A number of vegetable oils were initially screened and at different concentrations e.g. olive oil 5%, 10%, 20%, cottonseed oil, 5%, 10% and sunflower oil 5% and 10%. These oils were chosen due to their commercial availability and broad dietary use. A product containing 5% olive oil was originally the lead product, but it left a slight aftertaste and therefore oil that did not leave an unpleasant aftertaste was sought. Cottonseed oil also gave a bitter aftertaste, however sunflower oil left no appreciable aftertaste and also seemed to give a less oily texture to the product, than was provided by the olive oil. Sunflower oil is a common dietary product and therefore has no potential adverse side effects. A concentration of 5% was chosen as it was considered the lowest level that would provide adequate lubricating effect.

A second possible function of the sunflower oil in the formulation also presented itself. Research done in recent years has pointed to the use of essential fatty acids, such as those found in sunflower oil, being of use in rheumatological conditions. Three essential fatty acids, arachidonic acid (AA), dihomogammalinolenic acid (DGLA) and eicosapentaenoic acid (EPA) are precursor molecules of prostaglandins and other

eicasonoids, which are chemical regulators of inflammatory cell function. In general, the AA metabolites are harmful causing inflammation, thrombosis and vasospasm. In contrast, the products derived from DGLA and EPA e.g. PGE1, are anti-inflammatory, vasodilatory and anti-thrombotic. It is preferable therefore to raise levels of DGLA and EPA, which can be achieved by increasing concentrations of gamma-linolenic acid (GLA), which is rapidly converted to DGLA in the body. Sjögrens syndrome patients have been shown to have elevated linoleic acid concentrations in their red blood cells, but reduction below normal values for all metabolites, indicating a defect in metabolism. This defect results in inadequate conversion of linoleic acid to GLA, its first metabolite, suggesting that supplementation with GLA would be useful in treating Sjögrens syndrome (Horrobin, 1984). A limited number of studies have been carried out to look at the effect of increased GLA levels in rheumatoid conditions (Horrobin, 1986). Kunkel *et al.* (1981) supplemented rat's diet with evening primrose oil for 2 weeks before adjuvant challenge and demonstrated both a delay in the onset and a suppression of the polyarthritis response. In the formulation development associated with this project, the level of sunflower oil present is low and significant amounts of GLA would not be absorbed, however use of the product over long time periods, as is usual with a product for the treatment of xerostomia, may have some beneficial effect.

6.1.8 Bioadhesion

It is important that if the saliva substitute is to help in lubricating and protecting the oral cavity, it must be retained in the oral cavity for a prolonged period of time for its actions to be effective. The best way to ensure retention in the oral cavity is to use a bioadhesive polymer, which will adhere to the tissue in the oral cavity thus ensuring the preservation of the product for an increased period over what would be achieved in its absence. *Carbopol 974P*™, which is a polyacrylic acid, was chosen as the bioadhesive polymer of choice as polyacrylic acid polymers have been shown to have good mucoadhesive properties (Smart *et al.*, 1984). These polymers are currently not used in

any of the saliva substitutes available commercially in Ireland. *Carbopol 974P*™ is a lightly cross-linked polyacrylic acid that has been polymerized before recovery. The chemical structure of the *Carbopol*™ monomer can be seen in Figure 6.1.1. *Carbopol 974P*™ polymers are supplied as dry, tightly, coiled acidic molecules. Once dispersed in water the molecules begin to hydrate and partially uncoil. On conversion of the acidic polymer to a salt thickening occurs. *Carbopol 974P*™ polymer must be neutralized in order to achieve maximum viscosity. Unneutralized dispersions have an approximate pH range of 2.5 - 3.5 depending on the polymer concentration, with very low viscosities. Once a neutralizing agent is added e.g. triethanolamine, thickening gradually occurs, with optimum neutralization achieved at a pH of 6.5 - 7.0. The formulation developed has a pH of 6.75, so optimum neutralization of the gel has been achieved. Viscosity begins to decrease above pH 9.0 due to the dampening of the electrostatic repulsion caused by the presence of excess electrolytes (BF Goodrich).

Concentrations of neutralized *Carbopol 974P*™ (pH 6.75 ± 0.05) from 0.5% to 2.0% were examined in order to try and achieve the correct texture and consistency, while still maintaining bioadhesive ability. Both 2.0% and 1.5% were found to be too thick in texture, while *Carbopol 974P*™ 0.5% suffered a lack of permanence. A concentration of 1.0% was chosen as a suitable concentration as it showed good bioadhesion, but was also a suitable consistency for use in the oral cavity.

Carbopol 974P™ gel loses viscosity on exposure to sunlight, but is relatively unaffected by temperature variations. It is not subject to hydrolysis or oxidation and is resistant to bacterial growth. It is safe, non-toxic and tasteless. No primary evidence of allergic reactions has been observed in humans following topical application. It is not absorbed in the body and is excreted unchanged (Ahuja *et al.*, 1997). *Carbopol 974P*™ is currently used in a number of pharmaceutical preparations as a suspending agent, thickening agent and gelling agent, and is therefore pharmaceutically acceptable as a base for this formulation. BF Goodrich have a formulation on their website using

Carbopol 974P™ as a base for toothpastes, indicating its acceptance for use in products intended for the oral cavity.

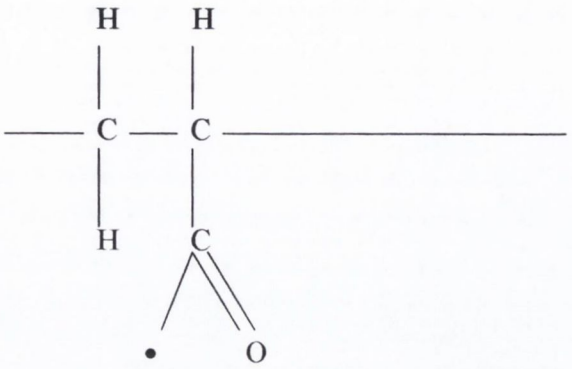


Figure 6.1.1 Monomer of polyacrylic acid (Ahuja *et al.*, 1997)

6.2 *In vitro* bioadhesion testing

6.2.1 Introduction

The term bioadhesion is used to define the attachment of synthetic or natural macromolecules to a biological substrate (Mortazavi, 1995). There are considered to be two principal mechanisms of bioadhesion;

(a) Formation of chemical bonds, including strong primary bonds (such as covalent bonds) and weaker secondary forces such as ionic bonds, van der Waal and hydrogen bonds. Covalent bonds are formed with proteins on the surface of epithelial cells. The other bond types are weaker, but when there are numerous interaction sites strong adhesions can be produced.

(b) Mechanical or physical bonds, macroscopically involving the inclusion of one substance in the cracks or crevices of another. On a microscopic scale, it involves the physical entanglement of mucin strands with flexible polymer

chains and/or interpenetration of mucin strands into a porous polymer substrate. The strength of the adhesive bond is directly proportional to the depth of penetration of the polymer chains.

There are a number of different methods that can be used to evaluate the bioadhesion of a polymer (Smart *et al.*, 1984, Ranga Rao and Buri, 1989, De Vries and Boddé, 1998, Chickering and Mathiowitz, 1995). The TA-XT2 texture analyzer used in this study was one that is relatively widely quoted in literature for its use in the measurement of bioadhesive strength (Caramella *et al.*, 1994, Wong *et al.*, 1999, Jones *et al.*, 1996). However it is not without its limitations. The instrument does not have a temperature control system and therefore experimental measurements are subject to temperature fluctuations. The results obtained are also highly dependent on a number of factors including the force applied, the probe used and the length of time the probe is left in contact with the sample (Wong *et al.*, 1999). As a result of these factors it is extremely difficult to make comparisons with literature that is currently available using this apparatus and the apparatus is only really suitable for direct comparison of systems, to provide relative bioadhesion values.

Bioadhesion testing was performed using three different substrates, fresh porcine oral mucosa (FPOM), fresh porcine gastric mucus (FPGM) and dried porcine gastric mucin (DPGM). The two parameters obtained from the adhesion testing were the peak detachment force (pdf) (mN) and the area under the curve (AUC), also known as the work of adhesion (WOA) (N.mm). Three substrates were used in order to examine the effect that the use of different substrates had on the results obtained and from this to provide information on the mechanism of bioadhesion that was occurring. Currently a problem with *in vitro* testing of bioadhesion is that a number of different substrates are used (Wong *et al.*, 1999, Mortazavi and Smart, 1995, Smart *et al.*, 1984, Caramella *et al.*, 1994) and there is no information regarding correlation or benefit of one over another.

6.2.2 Bioadhesion of *Carbopol 974P™*

The *Carbopol™* group of polymers are widely quoted as being good bioadhesive polymers (Wong *et al.*, 1999, Li *et al.*, 1998, Blanco-Fuente *et al.*, 1996a) and for this reason, along with their excellent safety profile, *Carbopol 974P™* was considered the bioadhesive polymer of choice for use in the development of a product for the treatment of xerostomia.

Carbopol 974P™ 1%, following a contact time of 60 s, showed the largest peak detachment force and work of adhesion with FPOM (Figure 6.2.1). There was a decrease of 20% in the peak detachment force and 16.7% in the work of adhesion with FPGM. There is yet a further decrease in both work of adhesion and peak detachment force with DPGM as substrate.

The bioadhesion over time of *Carbopol 974P™* 1% also varied with the substrate used (Figure 6.2.2). For FPOM an increase in both work of adhesion and peak detachment force was seen over time, while for FPGM a decrease in peak detachment force and no change in work of adhesion was observed. With FPGM there is a decrease in peak detachment force over time probably due to over hydration at the *Carbopol 974P™*/ mucus interface. This would not be seen with the FPOM, as it is not a liquid medium. DPGM showed little change in bioadhesion from 30 s to 60 s, but then showed a significant increase between 60 and 180 s. This would seem to suggest that whatever bioadhesive process is occurring with the DPGM is occurring at a much slower rate than seen with FPGM and FPOM. The DPGM is a crude mixture at a high concentration (25% w/w) and as a result may require a longer period for interfacial hydration and chemical bonding to occur.

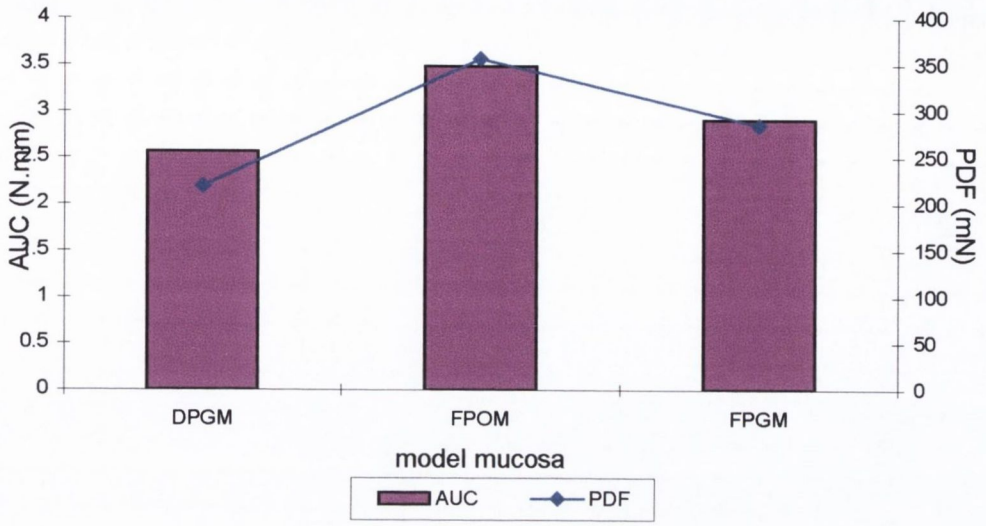


Figure 6.2.1 Bioadhesion of *Carbopol 974P*™ 1%, using three different substrates at 60 s.

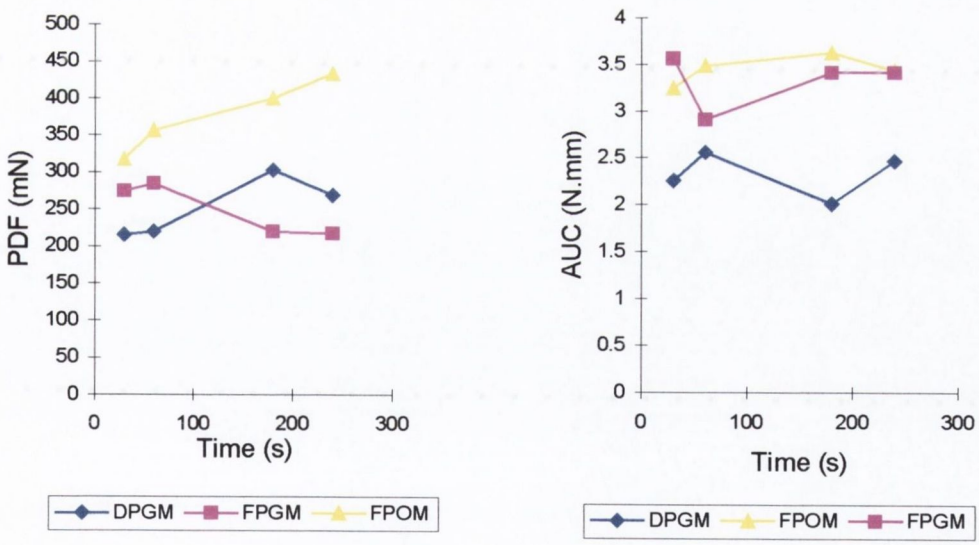


Figure 6.2.2 Bioadhesion of *Carbopol 974P*™ 1% over time using three different substrates.

The bioadhesive forces seen between FPOM and *Carbopol 974P*™ 1% are most likely due to a combination of chemical bonds, both strong covalent bonds with proteins on epithelial cell surfaces and weaker secondary forces such as van der Waal interactions and hydrogen bonds, and so conforms to the adsorption theory of bioadhesion (Section 1.10.6). The chemical bonding through van der Waal interactions and hydrogen bonding would be significant due to the large number of hydroxyl and carboxyl groups available for bonding, especially as the polymer is fully neutralized and therefore fully uncoiled exposing the maximum number of sites. The bioadhesive bond between FPGM, DPGM and *Carbopol 974P*™ 1% is less than with FPOM. This could be due to a number of reasons. Covalent bonding would not occur with FPGM, as there will be no epithelial proteins present. Therefore the bioadhesion will be due to secondary chemical bonding, which is not as strong. In order for this secondary chemical bonding to occur, polymer/mucus chain entanglement will need to take place. Due to the fact that the *Carbopol*™ polymer is full neutralized, the polymer chains will be fully expanded, possibly with some cross-linking occurring, resulting in few gaps where the mucin chains can penetrate.

6.2.3 Bioadhesion of product 12 and product 23

A number of the formulated products were examined for *in vitro* bioadhesion, including product 12 and product 23. Product 12 contained no oil. Product 23 contained 5% olive oil.

Product 12 gave the largest peak detachment force readings at 60 s for bioadhesion with DPGM, followed by FPOM and FPGM (Figure 6.2.3). However the work of adhesion values at 60 s did not follow the same trend with the greatest values being given by FPGM, followed by DPGM and then FPOM (Figure 6.2.3). Over time the peak detachment force of product 12 using FPOM showed a continual increase, there was a rapid decline in bioadhesion after 60 s with FPGM, and DPGM showed no appreciable

change over time (Figure 6.2.4). The result seen with FPGM points to there being a reduction in chemical bonding which is most likely due to the changed chemical conformation of the *Carbopol*™ polymer as a result of the presence of a large amount of ions. This will cause the polymer to partially recoil, leaving fewer sites exposed for secondary chemical bonding. The decrease over time is most likely due to over-hydration at the mucus/polymer interface causing secondary bonds to break down.

FPOM shows a smaller bioadhesive force at 60 s for the same reason as stated previously i.e. the changed chemical conformation of the polymer due to the presence of salt excipients. The increase over time with FPOM would point to the chemical bonding occurring more slowly with product 12 than with *Carbopol 974P*™ 1% (Figure 6.2.6). Once again this is most likely due to the bonding being slower due to the partially coiled nature of the polymer, making it more difficult for binding sites to be exposed.

The largest peak detachment force is seen with DPGM. This mucin showed an increase in bioadhesion for product 12 compared to *Carbopol 974P*™ 1%. The only possible explanation for this reverse trend is that a different method of bioadhesion is occurring with DPGM, perhaps mucin/polymer chain entanglement is becoming the predominant method due to the high concentration of mucin present. This is a possibility due to the increased space between the polymer chains as a result of the partial recoiling, leaving room for the mucin chains to penetrate.

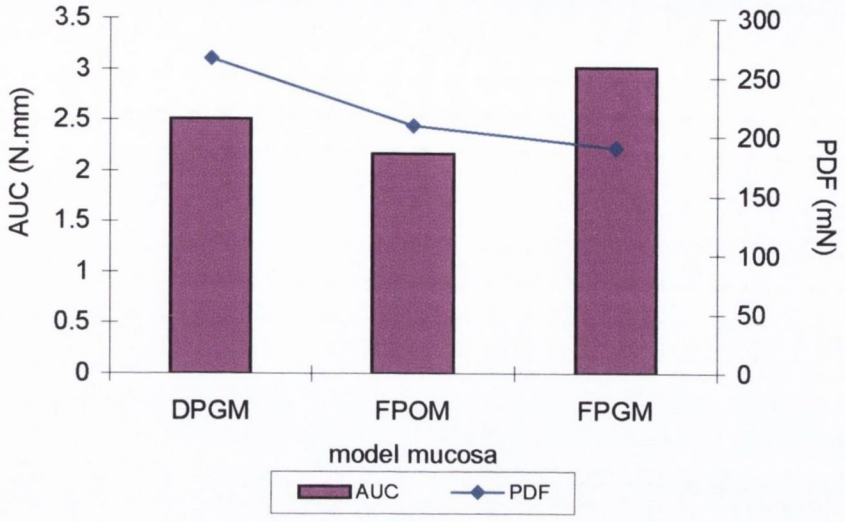


Figure 6.2.3 Bioadhesion testing of product 12 at 60 s using three different substrates.

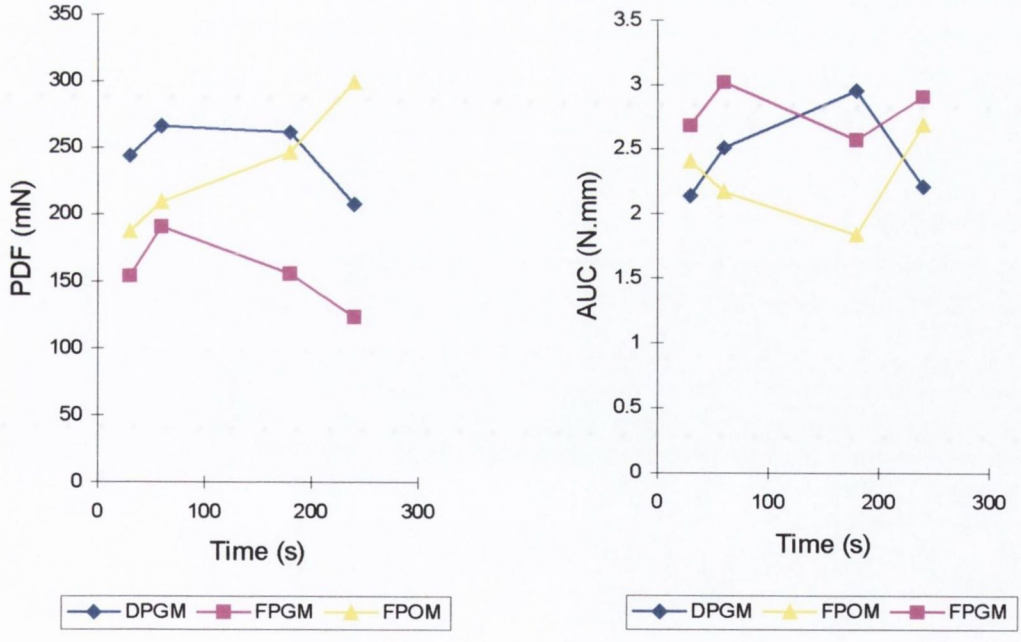


Figure 6.2.4 Bioadhesion of product 12 over time using three different substrates.

Product 23 showed a different pattern of bioadhesion at 60 s, with the largest bioadhesive force being given by FPGM, followed by dried porcine gastric mucus, with the weakest bioadhesive force being observed with FPOM, for both peak detachment force and work of adhesion (Figure 6.2.5). The bioadhesion seen with product 23 is greater than that observed with product 12 for all substrates. When bioadhesion of product 23 is examined over time, a rapid decline in bioadhesion is seen with FPGM after 60 s, there is little change observed over time with DPGM and there is a large increase in bioadhesion of product 23 with FPOM over time, more specifically between 180 s and 240 s, resulting in the FPOM showing the greatest peak detachment force at 240 s (Figure 6.2.6). The work of adhesion follows the same trend, however at 240 s all three substrates show approximately the same work of adhesion (Figure 6.2.6). The presence of the olive oil would appear to have a significant impact on the bioadhesive process. FPGM and DPGM show similar bioadhesive values, perhaps pointing to molecular chain entanglement playing a more important role here for the FPGM. The overhydration at the interface is still a problem with the FPGM resulting in a decline over time, however the bioadhesion is greater than for product 12 at 240 s. The FPOM shows a similar profile to product 12, with bonding occurring more slowly but still finally providing the strongest bond.

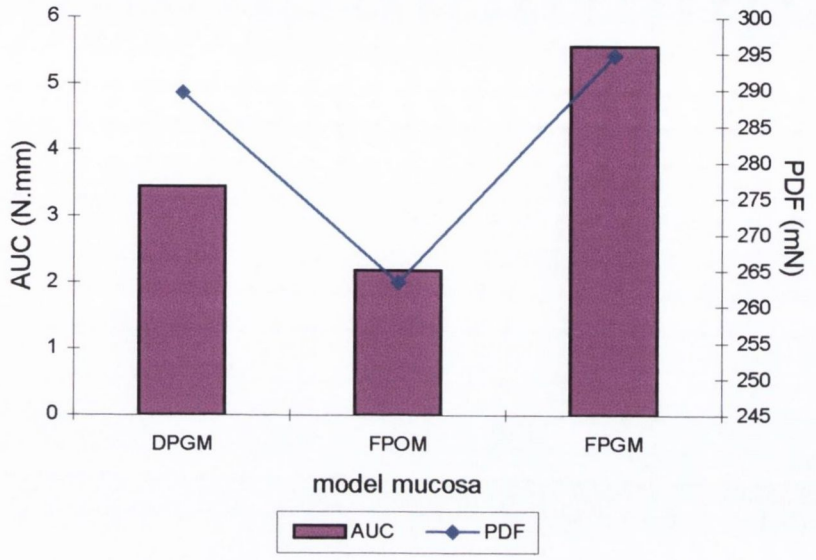


Figure 6.2.5 Bioadhesion of product 23 at 60 s using three different substrates.

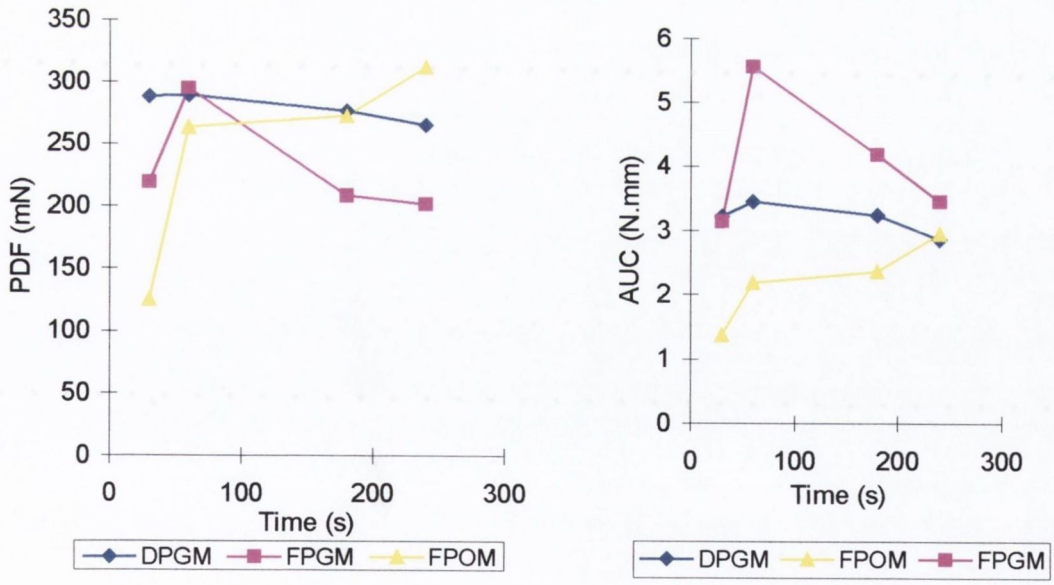


Figure 6.2.6 Bioadhesion testing of Product 23 over time using three different substrates.

The three substrates give different patterns of bioadhesion dependent on the product used, but they also give different results as to which system has the strongest bioadhesion. Overall however both *Carbopol 974P*™ 1% and product 23 show the strongest bioadhesion, with their bioadhesion being almost identical with FPGM and DPGM. The bioadhesion of *Carbopol 974P*™ 1% was significantly greater than product 23 when using FPOM. Product 23 at nearly all times and with all substrates showed greater bioadhesion than product 12. The reduction in bioadhesion of product 12 and product 23 compared to *Carbopol 974P*™ 1% is most likely due to the presence of salts in the formulation of products 12 and 23. *Carbopol*™ polymers have been shown to be highly sensitive to ions, with their presence affecting bioadhesion to mucus (Rossi *et al.*, 1995). In composition, the only difference between product 12 and product 23 was the addition of 5% olive oil to product 23. The presence of the oil appears to cause an increase in the bioadhesive force of the product, which could be due to a number of reasons. It could possibly cause a change in the chemical conformation of the polymer resulting in exposure of more binding sites. The presence of the oil could also help reduce overhydration at the mucus/polymer interface by acting as a barrier to penetration by water, resulting in a reduction in the speed at which hydration is able to occur.

6.2.4 Bioadhesion of products 27 and 28

The presence of the sunflower oil (5%) instead of the olive oil (5%) caused a small drop in bioadhesion. An increase in bioadhesion was seen when the sunflower oil was increased to 10% (product 28), however it was still less than that observed with product 23. A possible explanation for this change in bioadhesion is that structural changes could occur in the gel system due to the change in the oils being used, resulting in a weaker gel structure in the products using sunflower oil, with a resultant decrease in bioadhesion. This possibility is examined in Section 6.3.5.

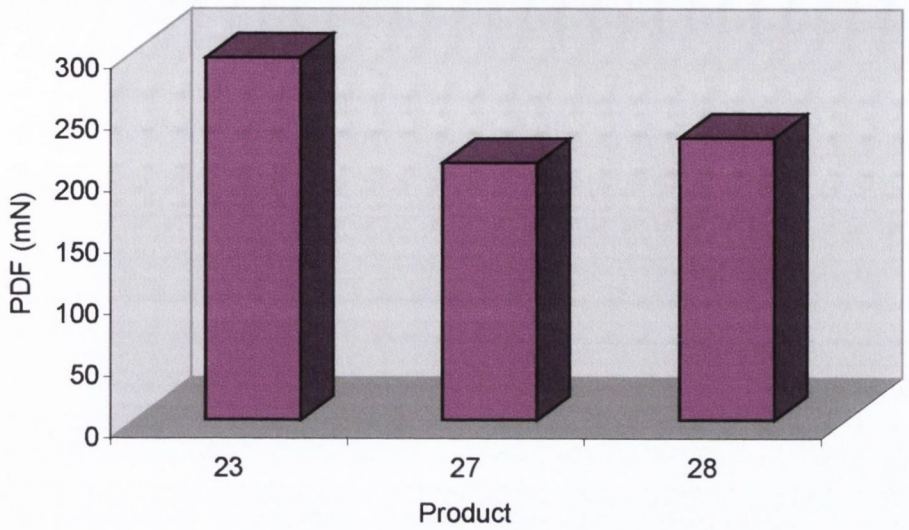


Figure 6.2.7 Comparison of products containing olive oil 5% (23), sunflower oil 5% (27) and sunflower oil 10% (28).

6.2.5 Factorial study of bioadhesion

A 3^3 factorial analysis was performed on the peak detachment force results obtained from the bioadhesion study to determine the significance of the various factors examined on the results achieved. Three factors were examined, each at three different levels as outlined in Table 6.2.1. The effect of the three variables and their levels on peak detachment forces (PDF) are summarized in Table 6.2.2. The results from the factorial analysis are outlined in Table 6.2.3. There are two main effects and two two-way interactions observed from the analysis.

Table 6.2.1 Levels examined in factorial analysis.

Factor	***Level 1***	***Level 2***	***Level 3***
Bioadhesive model	Fresh porcine oral mucosa (FPOM)	Fresh porcine gastric mucus (FPGM)	Dried porcine gastric mucin (DPGM)
Test substance	*Carbopol 974P™* 1%	Product 12	Product 23
Time (s)	60	180	240

Table 6.2.2 The effect of test substance, time of adhesion and bioadhesive model on PDF.

Bioadhesive model	***Time (s)***	***Test substance (PDF)***		
		Carbopol 974P™ 1%	***Product 12***	***Product 23***
FPOM	*60*	356.6	209.4	263.4
	180	400.4	246.6	272.8
	240	433.6	299.2	311.9
FPGM	*60*	285	190.8	294.8
	180	220.2	155.8	208.6
	240	217.4	123.4	201.8
DPGM	*60*	219.6	266.4	289.6
	180	303.6	261.6	276.6
	240	268.8	207.6	265.2

The two main effects are the bioadhesive model used and the test substance used, and their impact can be clearly seen in the main effects plot (Figure 6.2.8). In relation to the substrate used, FPOM gives the greatest overall peak detachment force while FPGM shows a reduction of 110 mN in comparison to FPOM, with DPGM giving a value intermediate between FPOM and FPGM. This result correlates well with other published data. Mortazavi (1995) found that mucus gel gave the weakest adhesive force on adhesion testing. Robert *et al.* (1988), also found that all bioadhesive polymers tested showed better adhesion on a mucin free epithelium than in the presence of mucus. The effect of product on peak detachment force shows product 12 giving a significantly lower value than either *Carbopol 974P*™ 1% or product 23, with product 23 showing 90% of the bioadhesion of *Carbopol 974P*™ 1%. The two-way interactions occurred between bioadhesive model and test substance and also between bioadhesive model and time.

Table 6.2.3 Analysis of variance of factorial study.

Source	***DF***	***Seq SS***	***Adj SS***	***Adj MS***	***F***	***P***
Bioadhesive model	2	166024	166024	83012	71.14	0.000
Test substance	2	108652	108652	54326	46.55	0.000
Time (s)	2	1678	1678	839	0.72	0.492
Bioadhesive model * product	4	45516	45516	11379	9.75	0.000
Bioadhesive model * time	4	72006	72006	18001	15.43	0.000
Test substance * time	4	10852	10852	2713	2.32	0.068
Bioadhesive model * product * time	8	16908	16908	2114	1.81	0.095
Error	54	63015	63015	1167		

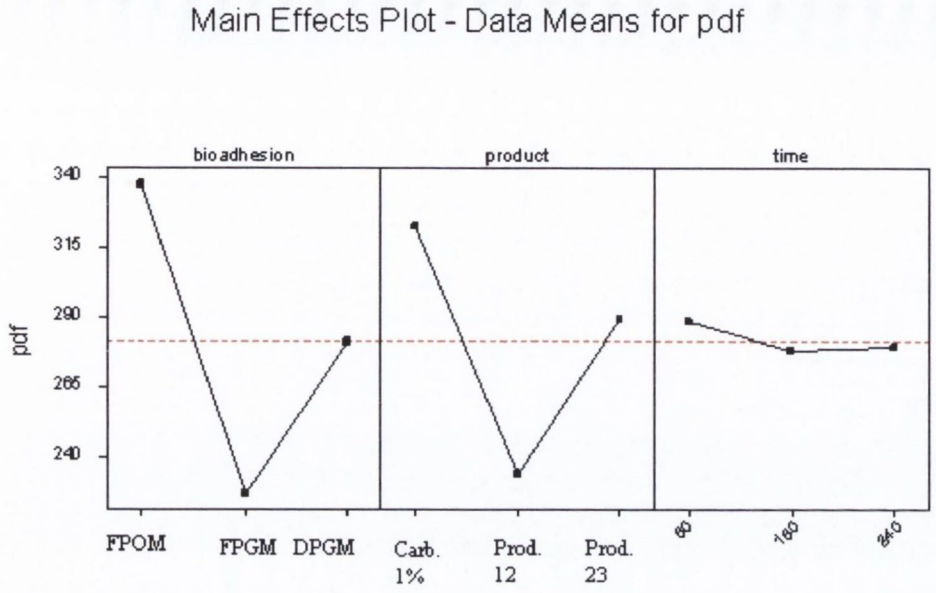


Figure 6.2.8 Main effect plots for bioadhesive substance, product and time.

The interaction plots (Figure 6.2.9) present us with a clear picture of the interaction between bioadhesive substrate and time, with FPOM giving an increase in peak detachment force over time, while FPGM causes a substantial decrease over time. As previously explained, the reduction in peak detachment force when using FPGM is most likely due to overhydration of the mucus/polymer interface. The other 2-way interaction was between bioadhesive substance and product, with product 12 showing a large decrease in peak detachment force when tested with FPOM and FPGM over the change seen when tested with DPGM.

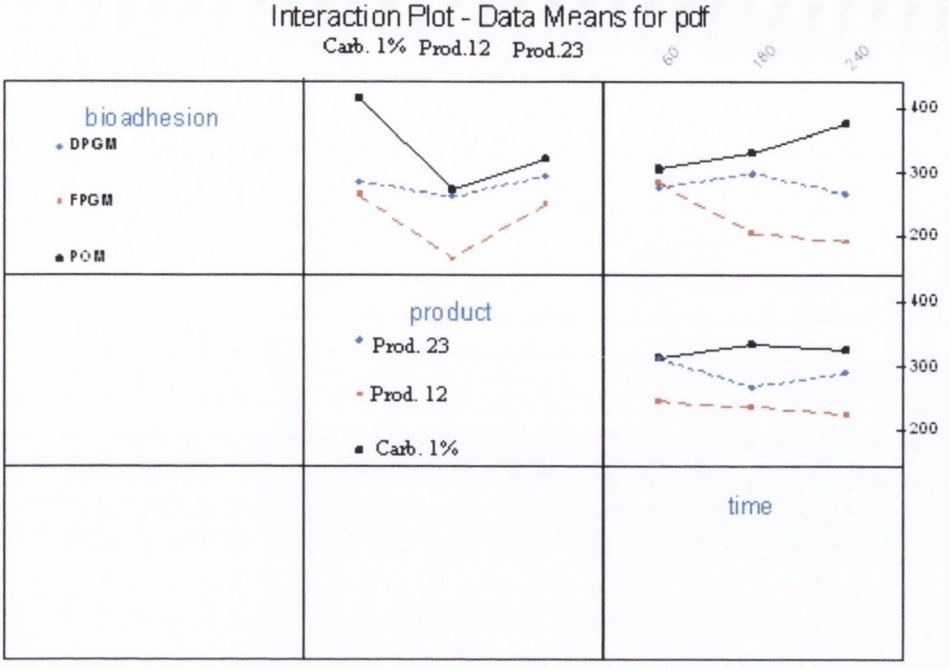


Figure 6.2.9 Interaction plots for test substance, bioadhesion model and time.

It is clear as a result of this study that the substance used, the time allowed for bioadhesion to occur and the product tested will all affect the results obtained. This variability is due mainly to changes in the mechanism of bioadhesion occurring and polymer conformation.

However in general the trends observed were similar. *Carbopol 974P*™ 1% is shown to be a good bioadhesive agent showing strong bioadhesion with all substrates used. It gives the strongest bioadhesion using FPOM, pointing to covalent chemical bonding being a significant factor in its bioadhesive process. The xerostomic formulations prepared did not have as strong a bioadhesive force as is seen with *Carbopol 974P*™ 1% alone, and show differing bioadhesive time profiles. Product 23, which contains olive oil 5%, shows considerably better bioadhesion with all substrates than product 12 (Table 6.2.2), which is probably due to the oil helping to provide a barrier at the interface of the mucus/polymer system so helping to prevent overhydration and also to it

providing a change in the conformation of the polymer allowing for greater exposure of binding sites. The fact that product 23 shows greater bioadhesion with FPGM than with FPOM is probably due to a change in the type of bioadhesion occurring, with interpenetration of mucin chains becoming a significant factor in the bioadhesive process, as well as chemical bonding.

6.3 Rheology

6.3.1 Introduction

Rheology is a method of classifying a gel, emulsion or other semi-solid systems by providing information on their structure. Oscillatory rheometry gives an indication of the rigidity and elasticity of the polymer network, while flow rheometry measures the apparent viscosity of the system. Oscillatory rheometry involves the application of an oscillatory shear stress to a sample and the subsequent measurement of the oscillatory shear strain. The parameters measured from this application of oscillatory shear stress are the storage modulus (G'), which is the measure of the energy stored and recovered per cycle of deformation and reflects the solid like or the elastic component of the viscoelastic behavior of the material, where there is complete recovery after removal of an applied stress. The loss modulus (G'') is a measure of the energy lost per cycle and reflects the liquid like component of the system, with irreversible deformation occurring after removal of an applied stress. The ratio of G' to G'' is known as tgδ and is a good indicator of how viscoelastic a system is, as it measures the lag in sine response after an oscillating mechanical field has been applied to a sample. A viscoelastic system is one that shows frequency dependence i.e. it responds differently to changing frequencies. There are a number of different classifications that can be used to differentiate different rheological types. The four most common are:

1. Dilute solution - shows larger G'' than G' over the entire frequency range, yet the moduli approach each other at higher frequencies.
2. Entangled network system - shows G'' and G' curves intersecting at the middle of the frequency range indicating a clear tendency for more solid-like behaviour at higher frequencies.
3. Weak gels - these have higher G' than G'' with the moduli running almost parallel to each other.
4. Strong gels - these also have higher G' than G'', however G' has a slope of approx. 0 and G'' displays a minimum at intermediate frequencies.

The G' and G'' values for a cross-linked gel would not be influenced by the frequency of oscillation, while a physically entangled gel network would show a substantial decline in G' at low frequencies. This is because in physically entangled systems macromolecules are given time to untangle and move past each other at low frequencies so that the material behaves more like a viscous liquid, while at higher frequencies they are only able to show elastic deformation. Intermediate behaviour suggests that the gel formed may be both an entangled and weakly cross-linked system held together by secondary chemical bonds (Mortazavi *et al.*, 1993).

6.3.2 Rheology of *Carbopol 974P*™

Both *Carbopol 974P*™ 1% and 0.5% show similar response over the frequency range, with a plateau response being achieved at approximately at 2 Hz for 1% and 4 Hz for 0.5%, continuing until the final result taken at 10 Hz (Figure 6.3.1). The storage modulus G' of *Carbopol 974P*™ 0.5% is seen to be half the G' for the 1% preparation throughout the frequency range, indicating that the G' in this particular *Carbopol 974P*™ grade would appear to be concentration dependant throughout the frequency range applied. The curves observed in Figure 6.3.1 are comparatively flat which according to Craig *et al.* (1994), indicates that cross-links or entanglements within the

gel prevent any substantial arrangement of the molecules, with the width of the plateau reflecting the degree of association within the gel. A plateau region that extends into the low frequency region reflects a highly cross-linked structure as is seen in the crossover of G' and G'' for 1% *Carbopol 974P*™, which occurs at a much lower frequency than in the 0.5% *Carbopol 974P*™, with the 1% plateau occurring before the 0.5%. It is obvious from the values given that the *Carbopol 974P*™ 1% is predominantly elastic, with the storage modulus being of an order of three times greater than the loss modulus at plateau frequencies. The *Carbopol 974P*™ 0.5% shows almost equal storage and loss moduli at plateau frequencies. The apparent viscosity of *Carbopol 974P*™ 1% at two different temperatures is also shown (Figure 6.3.2). The apparent viscosity profiles examined do not show any significant difference at the different temperatures, with both samples showing shear thinning.

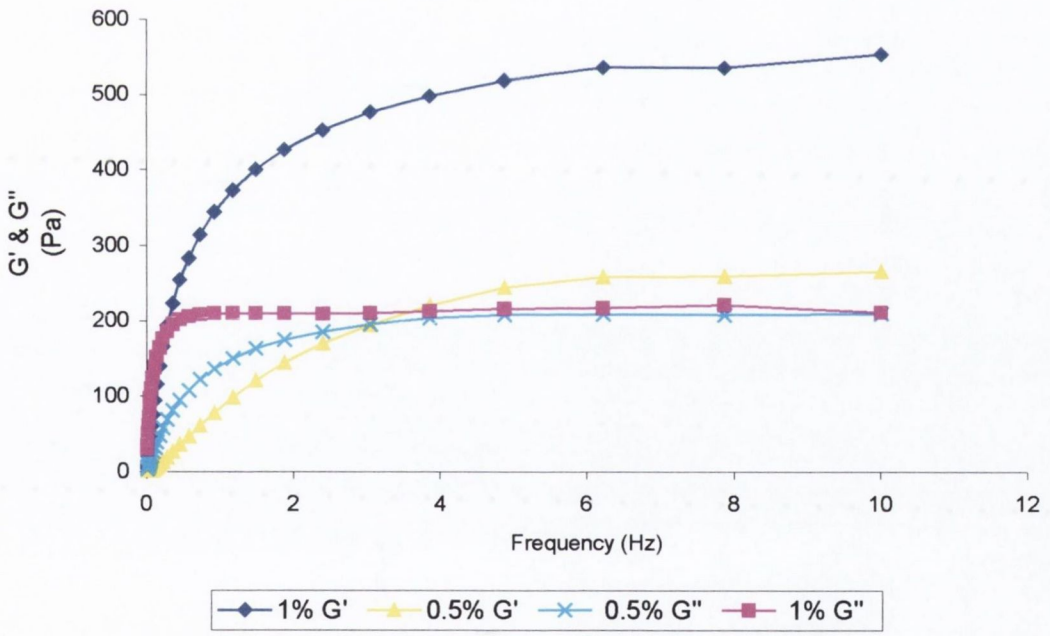


Figure 6.3.1 The storage and loss modulus of *Carbopol 974P*™ 1% and 0.5%.

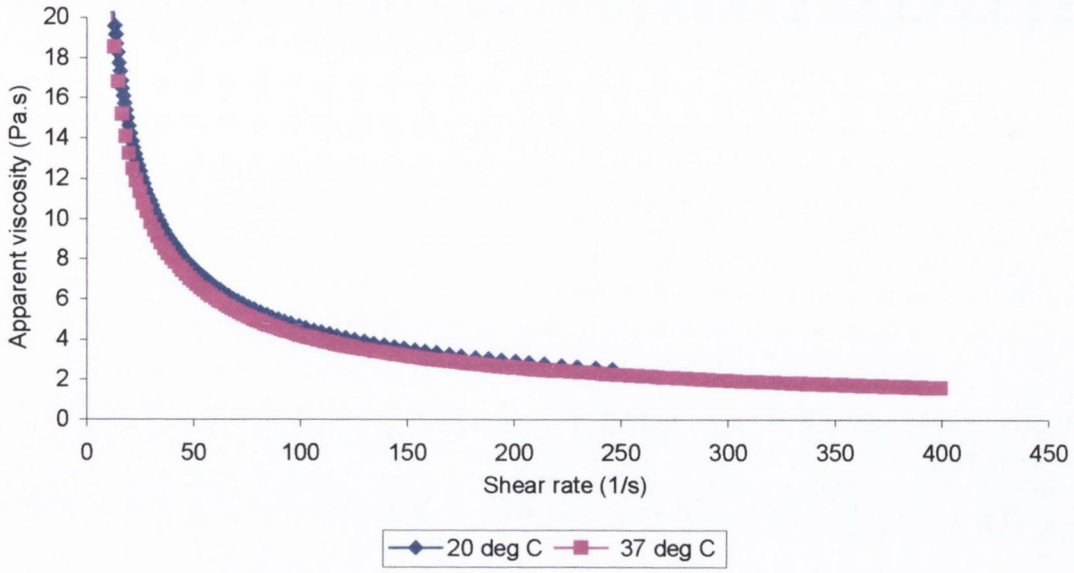


Figure 6.3.2 The apparent viscosity of *Carbopol 974P* 1% at two different temperatures.

6.3.3 Effect of addition of excipients

Product 12 and product 27 contain a number of added excipients, which are listed in Tables 3.8.2 and 3.8.3. The presence of these excipients cause a significant drop in the apparent viscosity of the system when compared to *Carbopol 974P*™ 1%. However the addition of the sunflower oil 5%, to product 27, which is the only difference from product 12, causes only a small difference in their apparent viscosities (Figure 6.3.3). This difference is more clearly seen in the shear rate *vs.* shear stress profiles, where a degree of thixotropy is seen to be present in the systems.

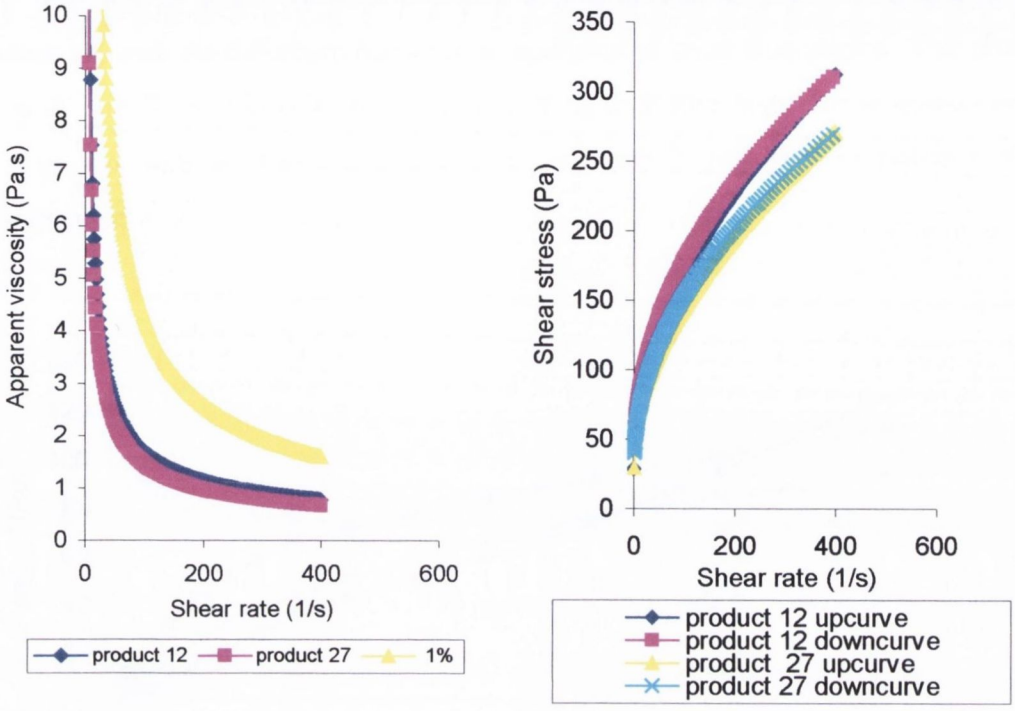


Figure 6.3.3 Apparent viscosity and shear stress/shear rate for *Carbopol 974P*™ 1%, product 12 and product 27.

It is apparent from Figure 6.3.4 that the inclusion of the excipients cause a significant change in the dynamic oscillatory rheology of the *Carbopol 974P*™ polymer system, with a large loss in the storage modulus and a change in the frequency dependence of the polymer. The storage modulus of product 12, which contains *Carbopol 974P*™ 1% shows a reduction too less than the storage modulus of *Carbopol 974P*™ 0.5%. The plateau effect seen with both *Carbopol 974P*™ 1% and 0.5% is not observed over the frequency range investigated with product 12 or product 23, with a gradual increase in the storage modulus being seen over the entire frequency range examined. The position of the intersection of the G' and G'' is also significantly changed, with an increase to much higher frequencies of between 8 and 9 Hz, depending on the particular formulation being examined. The loss modulus for product 12 is greater than the storage modulus over the frequency range up to 8.5 Hz, where a crossover occurs between the G' and

G''. Product 23 again shows a decrease in the storage modulus in comparison to product 12, with the difference being more significant at lower frequencies. This change in the G' and G'' would point to the system changing from a highly cross-linked system to a system with far less cross-linking and a much higher level of polymer chain entanglement.

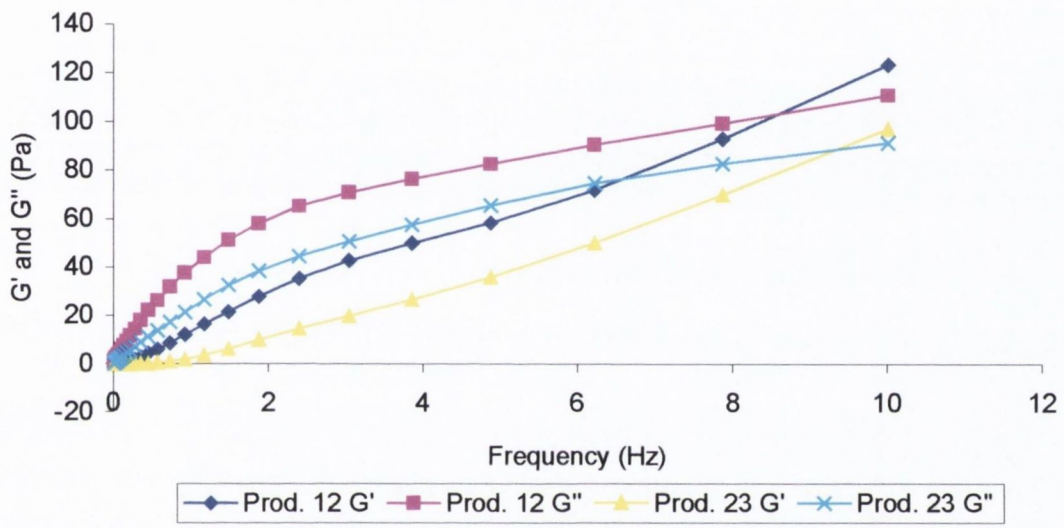


Figure 6.3.4 The storage and loss modulus of product 12 and product 23.

The presence of the salts in the formulation appear to have the largest impact, with their removal resulting in the largest increase in storage modulus, followed by the effect of removal of the sodium lauryl sulphate and triclosan (Figure 6.3.5). These results are to be expected, as the ionic nature of the salts will suppress electrostatic repulsion between molecules of the polymer resulting in it reverting to a more coiled form. This reduction in the expanded nature of the polymer will prevent the polymer chains coming into contact with one another and so will prevent contact occurring between polymer chains. This results in a change to the rheological profile with a lower storage modulus and a higher loss modulus, as the system is not as strong in the absence of cross-linking.

The removal of the xylitol and sorbitol causes a reduction in the storage modulus, implying that the sweeteners contribute to the conformational structure of the polymer. This could be due to the large quantity of sweetener present, which acts to increase the structural integrity of the system. Product 12 can be seen to have a slight up curve over the frequency range 0.1-4 Hz, with the curve flattening out above this point. This curvature over the lower frequency range is pronounced in the absence of salts, sodium lauryl sulphate and triclosan. This particular response disappears completely in the absence of xylitol and sorbitol, and also appears to be negated by the presence of the olive oil at a concentration of 5%.

The presence or absence of the various excipients also has a pronounced effect on the intersection point of the storage and loss moduli (Figure 6.3.6). The absence of the sweeteners xylitol and sorbitol, while causing a reduction in the storage modulus does not affect the cross-over point between G' and G'', so they would not appear to have an impact on the polymer conformation. In the absence of sodium lauryl sulphate, triclosan and the salts, the intersection point shifts significantly downwards to 5 Hz approx., with the storage and loss moduli almost equal in profile up to that point. This would signify that in the absence of sodium lauryl sulphate, triclosan and the salts, the polymer is present in a more expanded form with a greater level of polymer chain entanglement occurring. This is due to the fact that the presence of these excipients causes the polymer to adopt a more coiled form, resulting in less polymer entanglements.

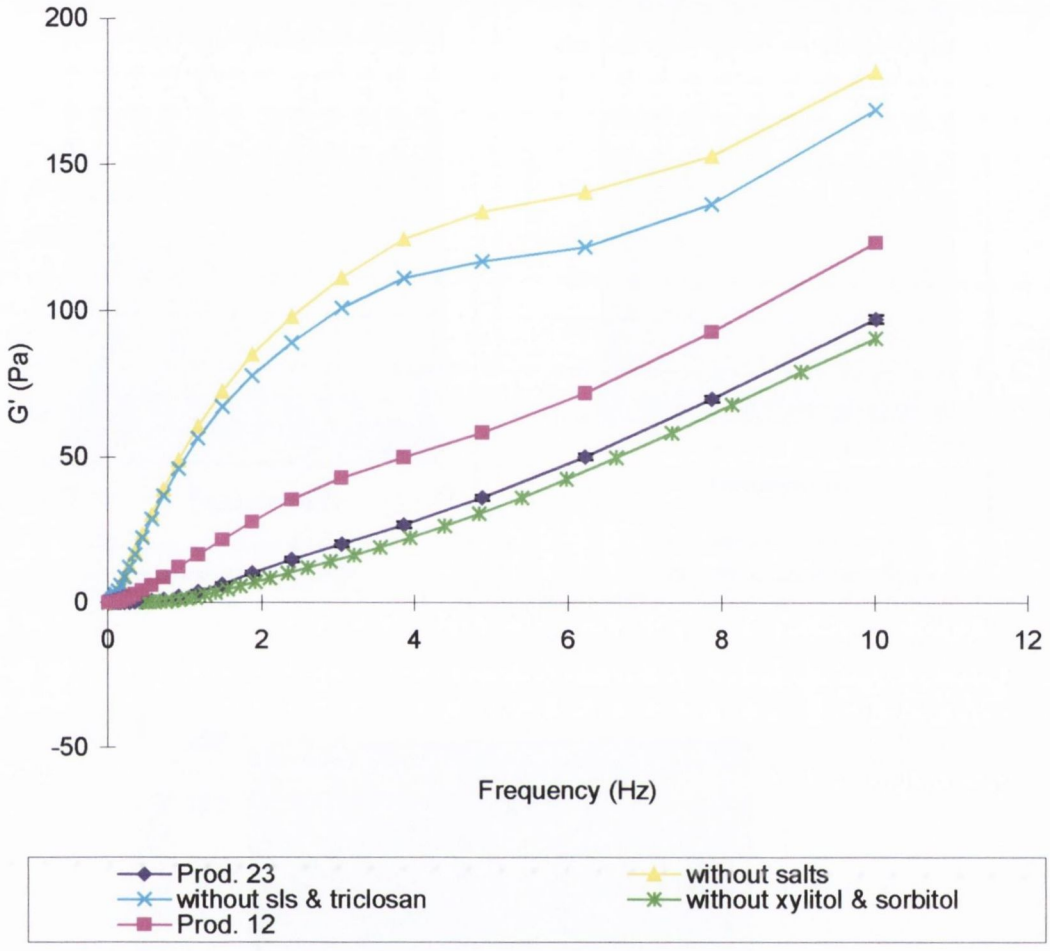
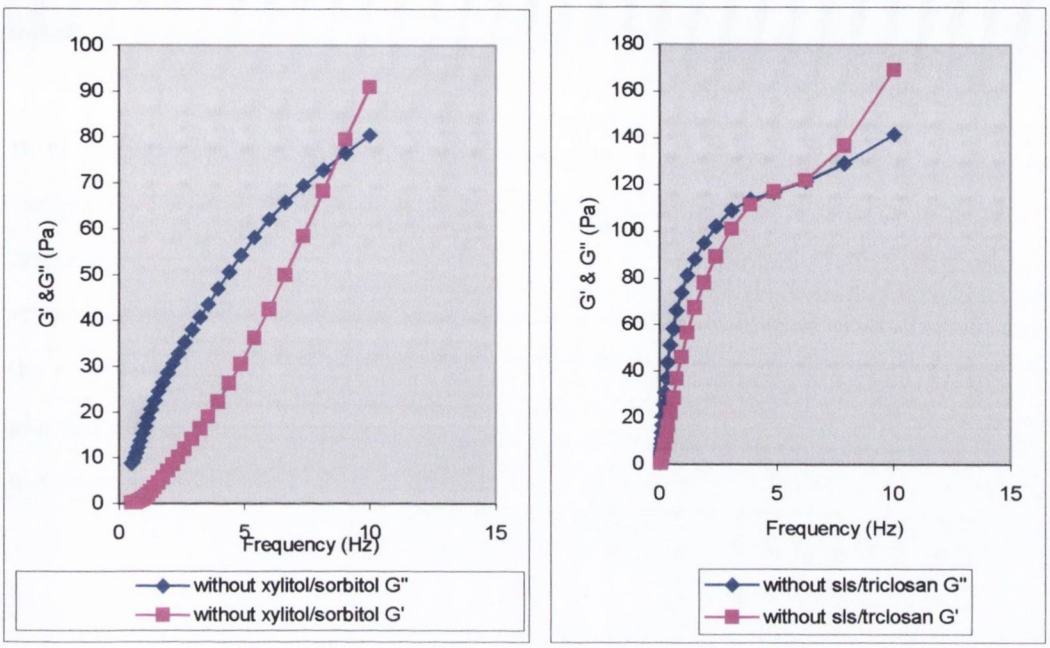


Figure 6.3.5[1] The effect of various excipients on the storage modulus of Product 12.

[1] Without salts: Product 12 without sodium chloride, calcium chloride, potassium chloride, magnesium chloride, potassium phosphate, sodium fluoride.

Without xylitol and sorbitol: Product 12 without xylitol, sorbitol and lemon flavoring

Without sodium lauryl sulphate and triclosan: Product 12 without triclosan and sodium lauryl sulphate



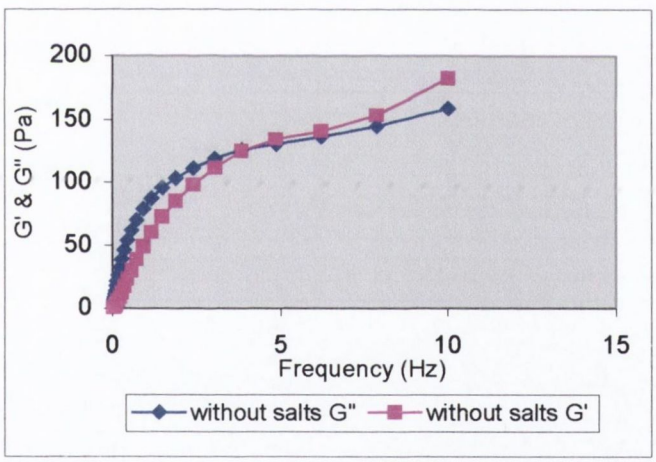


Figure 6.3.6[1] The effect of excipients on the storage and loss moduli.

6.3.4 Correlation between oscillatory rheology and bioadhesion

It is evident from Figure 6.3.5 and Figure 6.3.6 that the addition of the various excipients have a profound effect on the rheology of the *Carbopol 974P*™ polymer dispersion, with significant changes in the storage modulus, loss modulus and overall viscoelasticity being observed (Table 6.3.1). The strong viscoelastic response seen with the *Carbopol 974P*™ 1% is far less evident in both product 12 and product 23, with the plateau effect observed in *Carbopol 974P*™ 1% from frequencies of 5 Hz and upwards not present in product 12 and product 23.

	G' (Pa)	*G'' (Pa)*	*tgδ*
Carbopol 974P™ 1%			
0.01 Hz	4.974	29.09	10.848
1.0 Hz	373.333	210.47	0.5845
10.0 Hz	554.196	211.57	0.4773
Product 12			
0.01 Hz	0.0084	0.540	299.1
1.0 Hz	16.5	44.12	2.964
10.0Hz	124	111.25	0.899
Product 23			
0.01 Hz	0.894	0.2578	33.56
1.0 Hz	4.02	26.117	7.844
10.0 Hz	96.1	89.03	0.928

Table 6.3.1 Representative values for *Carbopol 974P*™ 1%, product 12 and product 23.

The relevance of these changes in oscillatory rheology is of considerable significance in relation to the trends observed during bioadhesion testing. It is clear from Figure 6.3.7 and Figure 6.3.8 that the lower the tgδ, the greater the bioadhesive force achieved. This would point to elasticity being an important factor in determining bioadhesion, which has been previously quoted in the literature (De Vries and Boddé, 1998). It must be recalled however that *Carbopol 974P*™ 1% showed a reduction in bioadhesion with FPGM when compared to FPOM (Figure 6.2.1), and that the inverse of this was seen with product 12 (Figure 6.2.3) and product 23 (Figure 6.2.5), showing a greater bioadhesion with FPGM compared to FPOM. It is proposed that the reduction in bioadhesion seen with *Carbopol*™ 1% when using FPGM could be due to a lack of interpenetration of polymer and mucus chains with resultant secondary chemical bonding, due to the expanded nature of the polymer. This expanded polymer would result in the polymer chains coming in contact with one another causing cross-linking and chemical bonding between the polymer chains.

The results obtained here further support that theory. Figure 6.3.4 clearly shows that *Carbopol 974P*™ 1% is a strongly cross-linked and entangled system which would make it difficult for mucin chains to penetrate into the polymer. Figure 6.3.5 and Figure 6.3.6 show the reduced level of cross-linking due to the presence of the excipients as they cause the polymer chains to revert to a partially coiled form. This will allow for interpenetration and entanglement of polymer and mucus chains, which explains the increased bioadhesion seen with FPGM for product 12 and product 23, when compared to FPOM.

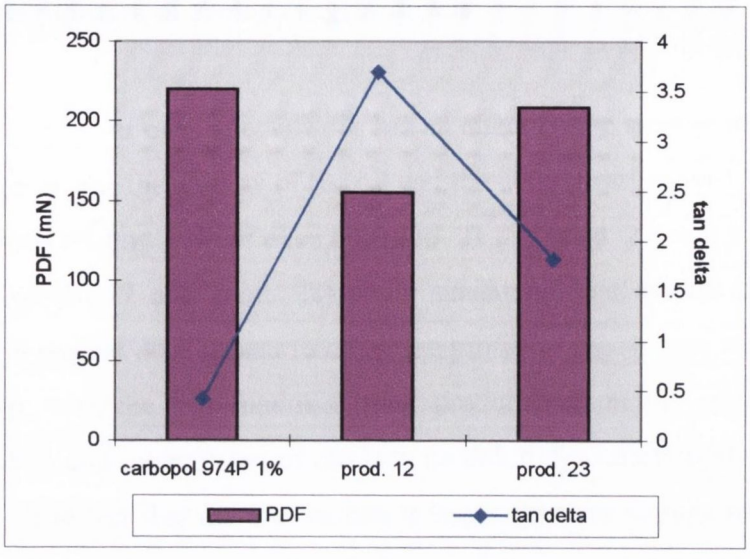


Figure 6.3.7 Relationship between tgδ and PDF for fresh porcine gastric mucus.

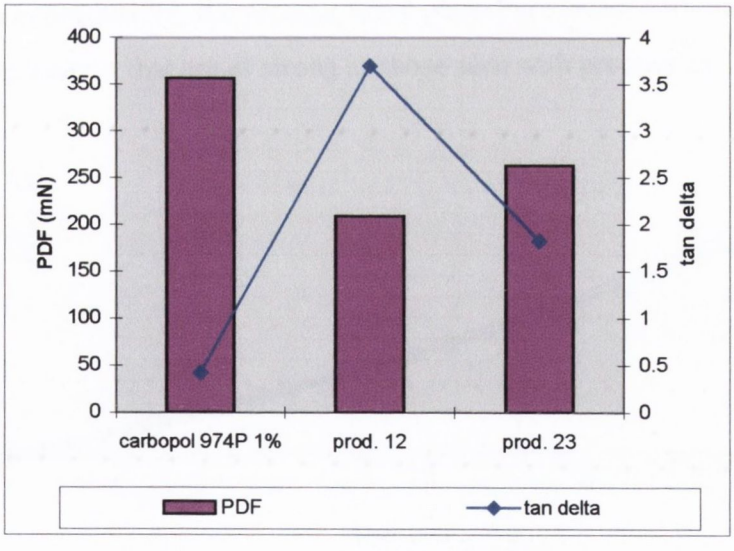


Figure 6.3.8 Relationship between tgδ and PDF for FPOM.

6.3.5 Effect of addition of various vegetable oils

While the addition of various vegetable oils has an effect on the storage modulus, as has been shown previously, the identity of the oil and the concentration used appears not to be a factor. Both 5% and 10% of olive oil (prod. 21 and prod. 23) and 5% and 10% of sunflower oil (prod. 27 and prod. 28) show similar storage moduli (Figure 6.3.9). However the oil present does appear to effect the profile of the storage *vs.* loss modulus (Figure 6.3.10), with the loss modulus being predominant in the system containing sunflower oil 5%, and intersection of the two moduli only occurring at frequencies of approx. 9 Hz. The fact that the loss moduli is larger than the storage moduli over the greater part of the frequency range, and that no frequency dependence is observed, would suggest the properties of a weak gel, with less entanglement than product 23. This could help explain the lower value for bioadhesion observed for product 27, compared to product 23 when using FPGM (Figure 6.2.7). Due to the weaker nature of the gel system in product 27, the product when placed in contact with the FPGM would not form entanglements that are as strong as those seen with product 23.

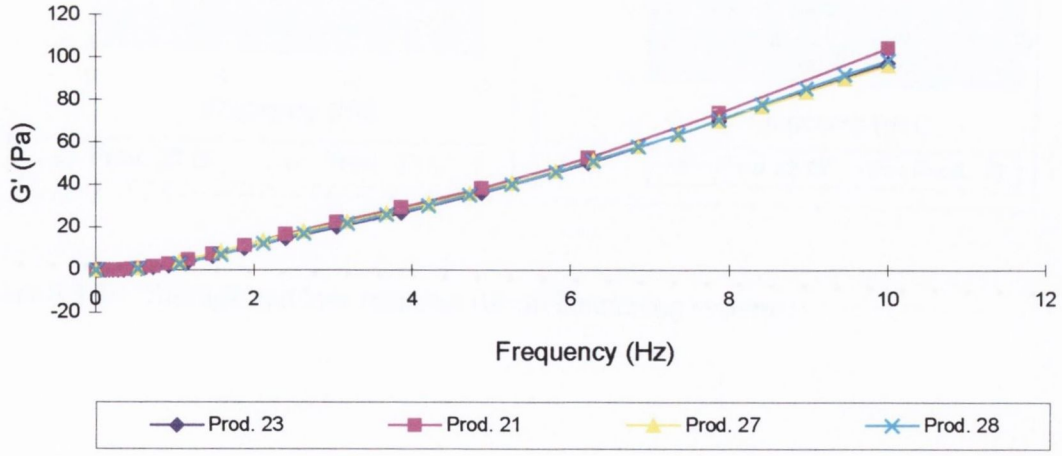


Figure 6.3.9 The storage moduli of products containing varying concentration of two different oils.

Overall the rheology of the final product, product 27 containing 5% sunflower oil, is very different in terms of structure from the gel base, *Carbopol 974P*™ 1%, with the different excipients having a profound effect on the structure of the gel. The presence of the excipients causes the polymer molecules to partially recoil to different extents, resulting in a gel that has polymer chain entanglement to account for its structural integrity. This is different to the base gel, which was fully expanded and with polymer chains in contact with one another resulting in cross-linking, this gave a strong gel system, shown in the high storage moduli seen with *Carbopol*™ 1%.

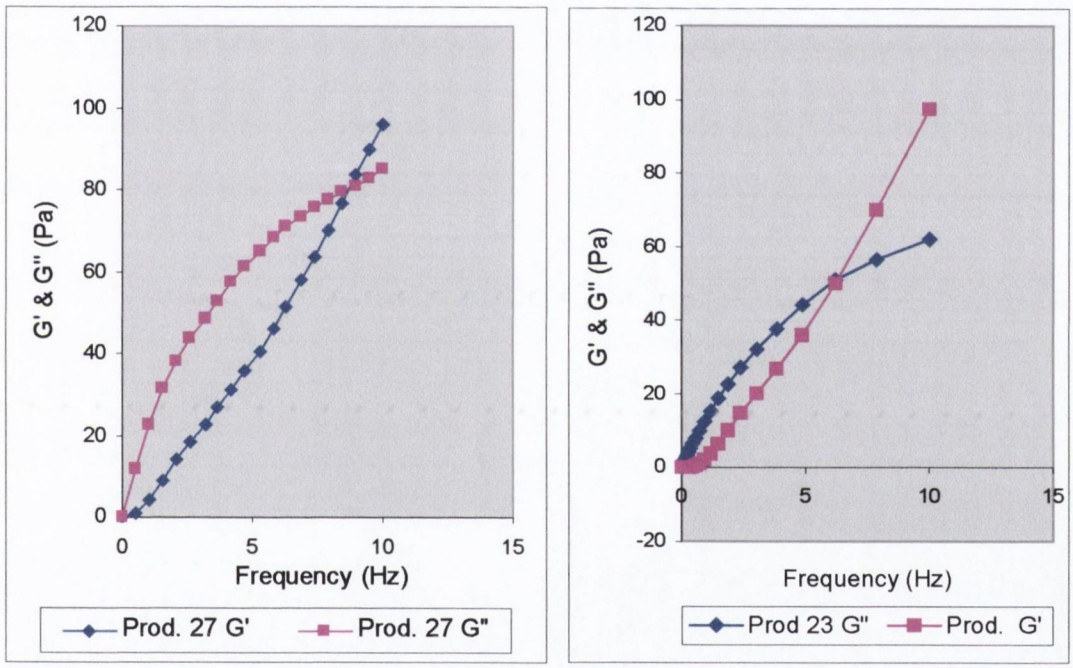


Figure 6.3.10 Storage and loss modulus for oil containing systems.

6.4 Rheological synergism

6.4.1 Introduction

Rheological synergism is widely used to examine the mucoadhesive properties of polymers. The method simulates the interpenetration layer in the mucoadhesion process (Hägerström *et al.*, 2000). This is a proposed mechanism of mucoadhesion where the mucus and the mucoadhesive molecules become firstly entangled and secondly form non-covalent bonds (Mortazavi and Smart, 1994). Dûchene *et al.* (1988) have proposed the following stages in the process of mucoadhesion. The first stage involves the establishment of an intimate contact between the mucoadhesive polymer and the mucus gel. Next the mucoadhesive polymer can penetrate the mucus gel network, resulting in the formation of physical chain entanglements and secondary chemical bonds between the mucus gel and the mucoadhesive material. *Carbopol 974P*™ 1%, product 12 and product 23 were all examined for rheological synergism. An increase in the elastic modulus for the mixture compared to the polymer alone is considered as a positive interaction caused by mucoadhesion.

It was found also that for polyacrylic acid, rheological interaction was strongly influenced by mucin type, probably due to its sensitivity to ions (Rossi *et al.*, 1995). FPGM was used in the experiments performed due to the relative ease with which it could be obtained. However it was extensively dialyzed prior to use to minimize the impact that ions might have on the rheological interaction.

6.4.2 Rheological synergism of *Carbopol 974P*™

The systems examined are outlined in Table 3.12.4. The *Carbopol 974P*™ 1%/mucus (system 3) at a representative frequency of 1 Hz showed no change in G' and a

reduction in G'' (Table 6.4.1). This resulted in a reduction in the tgδ, pointing to the system becoming more elastic in structure (Table 6.4.2). Figure 6.4.1 shows the G' and G'' of system 3 and system 7 (*Carbopol 974P*™ 1%/water) over a frequency range of 1 – 10 Hz. It is evident that there is a significant drop in the G' and G'' in system 3 compared to system 7. The frequency at which G' and G'' intersect is dramatically lowered by the presence of the mucus, pointing to a change in the physical conformation of the system. The reduction in G' for *Carbopol*™ has also been observed by Hägerström *et al.* (2000) and Rossi *et al.* (1995), where a negative interaction was seen with *Carbopol 974P*™ gel in water and different hydration media. Caramella *et al.* (1994) also observed a lack of a positive interaction with *Carbopol 974P*™ 1%. *Carbopol 974P*™ 1% has a fully expanded structure and a high level of cross-linking as is shown in Figure 6.4.1 and the interaction with mucin did not appear to significantly change the overall elastic structure of the system.

Table 6.4.1 Dynamic viscoelastic parameters (at 1 Hz frequency) for polymers and polymer/mucus mixtures.

	Polymer alone			***Polymer/mucus mixture***		
	G' (Pa)	***G'' (Pa)***	***tgδ***	***G' (Pa)***	***G'' (Pa)***	***tgδ***
Carbopol 974P™ 1%	75.45 ± 23.17	139.55 ± 45	1.908 ± 0.38	74.69 ± 15.9	77.82 ± 12.9	1.05 ± 0.12
Product 12	0.059 ± 0.005	2.1 ± 0.52	32.47 ± 1.54	7.15 ± 0.08	13.175 ± 0.06	1.84 ± 0.1
Product 23	0.033 ± 0.001	1.508 ± 0.09	47.915 ± 0.64	5.68 ± 1.08	11.64 ± 1.28	2.067 + 0.17

Table 6.4.2 Difference in viscoelastic parameters between polymers and polymer/mucus mixtures.

	ΔG'	***ΔG''***	***Δtgδ***
Carbopol 974P™ 1%	- 0.76	- 61.73	- 0.89
Product 12	7.09	11.08	- 30.63
Product 23	5.65	10.13	-45.85

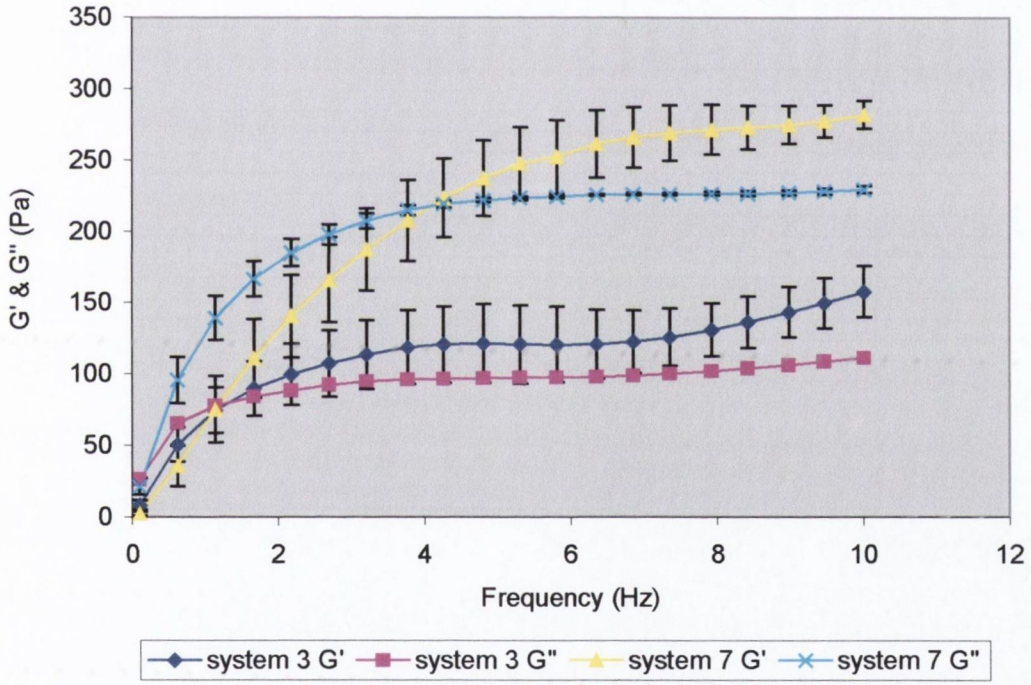


Figure 6.4.1 Rheological synergism of *Carbopol 974P™ 1%*/mucus (system 3) compared to polymer alone (system 7).

6.4.3 Rheological synergism of product 12 and product 23

A strong positive interaction was observed for product 12 (Figure 6.4.2) and product 23 (Figure 6.4.3), with a significant increase being seen in the storage modulus of both when mixed with mucus. The difference in the tgδ is large (Table 6.4.2), pointing to the systems having a pronounced increase in their elastic properties. This is considered to be due to interpenetration and secondary chemical bonding occurring between the system and the mucus. Product 23 shows a greater increase in its elasticity (Table 6.4.2).

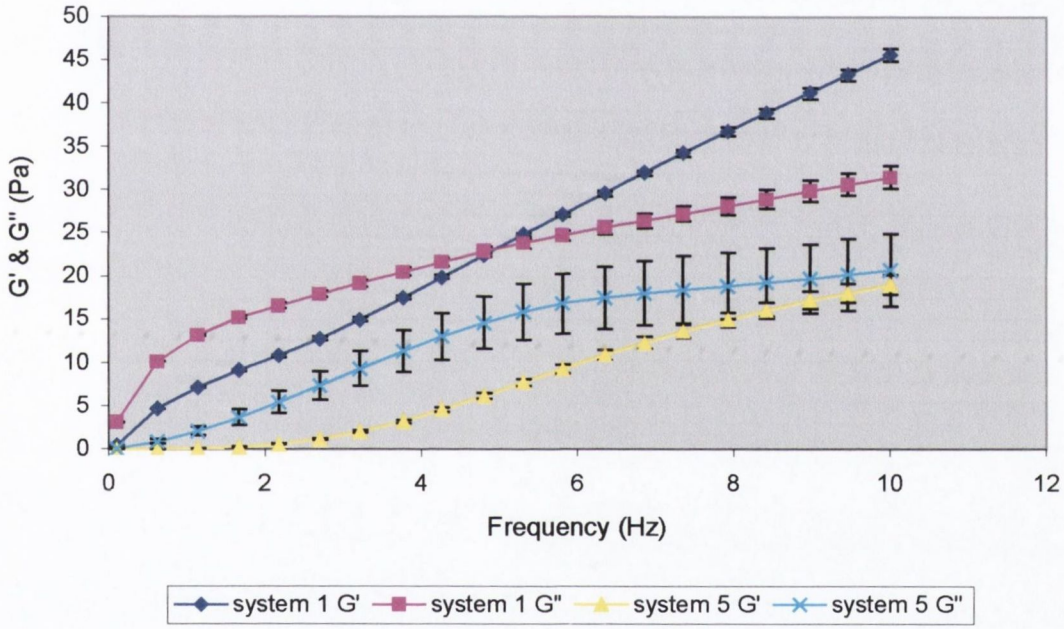


Figure 6.4.2 Rheological synergism of product 12/mucus (system 1) compared to product 12 alone (system 5).

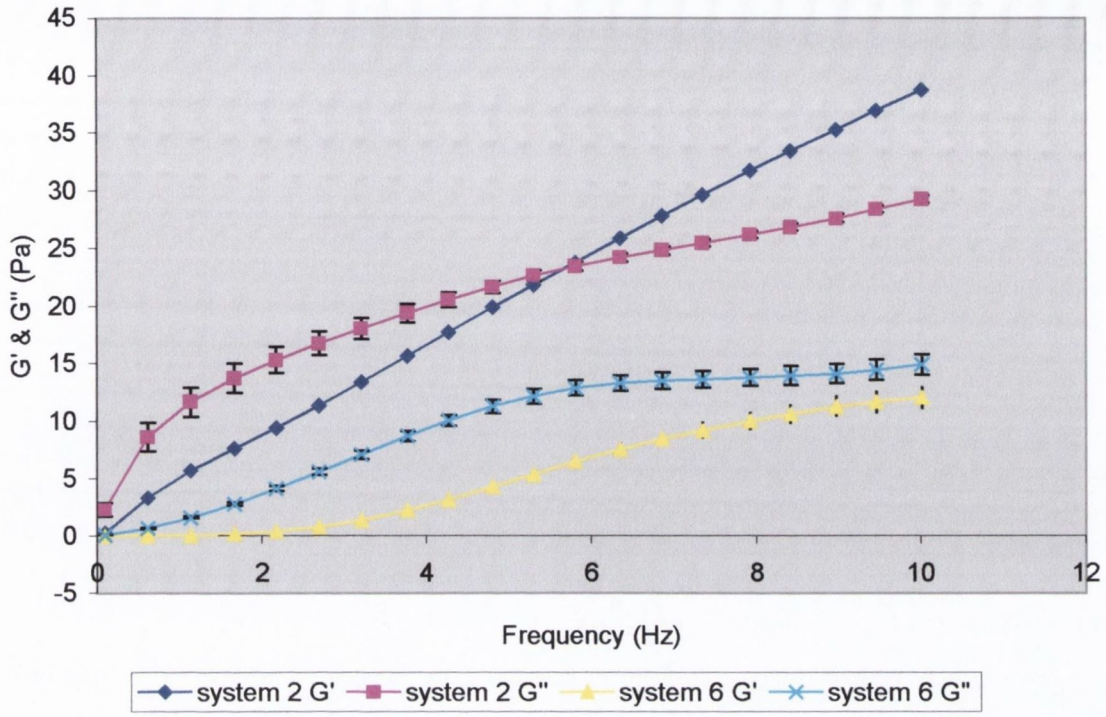


Figure 6.4.3 Rheological synergism of product 23/mucus (system 2) compared to product 23 alone (system 6).

6.4.4 Correlation between rheological synergism and tensile testing

No correlation was found between results from tensile testing and rheological synergism. Linear regression analysis was performed on multiple variables including tgδ, Δtgδ, pdf and AUC, with no relationship found between them. However there were some general trends that could be drawn between the two sets of data. *Carbopol 974P*™ 1% showed strong bioadhesive properties with FPOM using tensile testing, however its bioadhesion with FPGM was significantly lower (Figure 6.2.2). The strong bioadhesion seen with FPOM was considered to be a result of covalent bonding with proteins in mucosal epithelium as well as due to secondary chemical bonding. Bioadhesion with FPGM would occur due to interpenetration and secondary chemical bonding. The results from

the rheological synergism point to *Carbopol 974P*™ 1% not having a strong interaction with mucus (Figure 6.4.1), which would correlate with results from tensile testing. The reason for this lack of interpenetration of mucus with polymer is not clear, but it could possibly be due to the fact that the polymer chains are fully neutralized and therefore fully expanded. The fully expanded polymer chains would come in contact with one another and form cross–links between each other through chemical bonding. This cross–linked structure of the polymer would prove to be a difficult environment for the mucus chains to penetrate into as there would be few gaps in the polymer structure. The polymer would also be less inclined to penetrate into the mucus as its chains would be cross–linked to one another. As a result of these factors chain interpenetration and entanglement would not be a significant mechanism of bioadhesion here.

Product 12 and product 23 both showed improved bioadhesion with FPGM and DPGM compared to FPOM when using tensile testing (Figure 6.2.3, Figure 6.2.5). When the results obtained from rheological synergism are examined there is good agreement with the results from tensile testing, as a strong rheological synergy was observed with product 12 and product 23. The reason for this improvement in bioadhesion when using FPGM and DPGM is most likely due to a change in the conformation of the polymer, due to the presence of electrolyte. Product 12 and product 23 both contain significant amounts of salts. *Carbopol 974P*™ in the presence of salts will lose its fully expanded structure and begin to recoil. As a result of this the polymer chains will not be fully expanded and therefore it will be easier for mucin chains to penetrate the polymer, allowing for entanglement and the formation of secondary chemical bonds.

6.5 Friction testing

6.5.1 Introduction

A major problem associated with salivary lubricants is the fact that they are often too tacky and give an unpleasant feeling of stickiness in the mouth. This is often due to the nature of the bioadhesive polymer used in the formulation, which gives good oral adhesion but tends to dehydrate over time leaving a tacky feeling in the mouth. In an effort to address this problem in the formulation of the product, it was decided to incorporate a vegetable oil into the formulation. It was hoped that the oil would help reduce the level of tack in the product and so provide a better feeling of lubrication in the oral cavity. Friction testing was performed to provide an estimate of the lubricity of certain products being formulated.

6.5.2 Results of friction testing

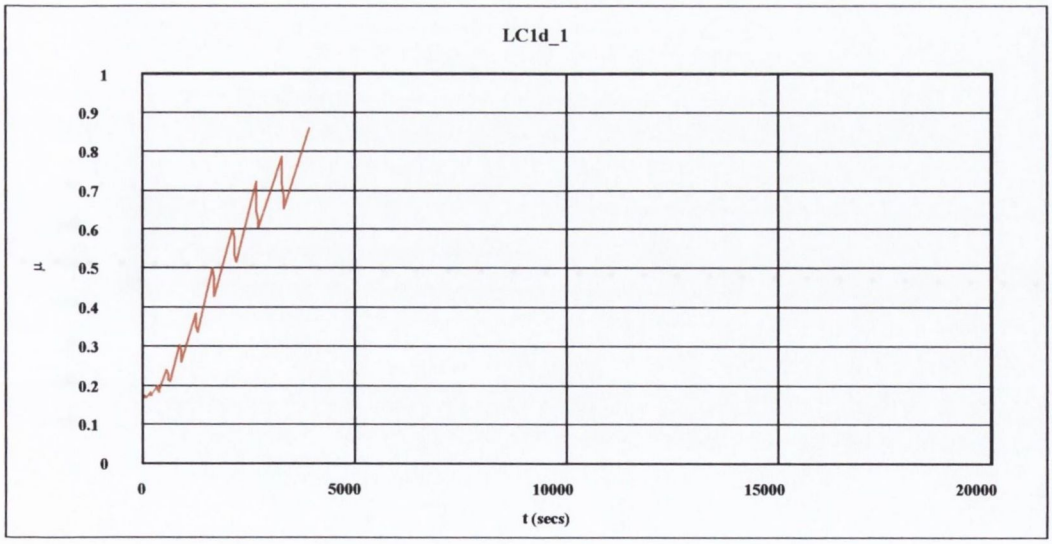


Figure 6.5.1 Lubricity testing of *Carbopol 974P*™ 1%.

It is clear from Figure 6.5.1 that the friction coefficient of *Carbopol 974P*™ increases rapidly with time. It reaches 0.5 at 40 min. This is most likely due to dehydration of the polymer dispersion over time, resulting in the polymer dispersion becoming more concentrated, stickier and therefore having a greater friction coefficient. This high friction coefficient would tend to give an unpleasant feeling and consistency in the oral cavity. Therefore it is necessary to modify the polymer system to produce a lower friction coefficient.

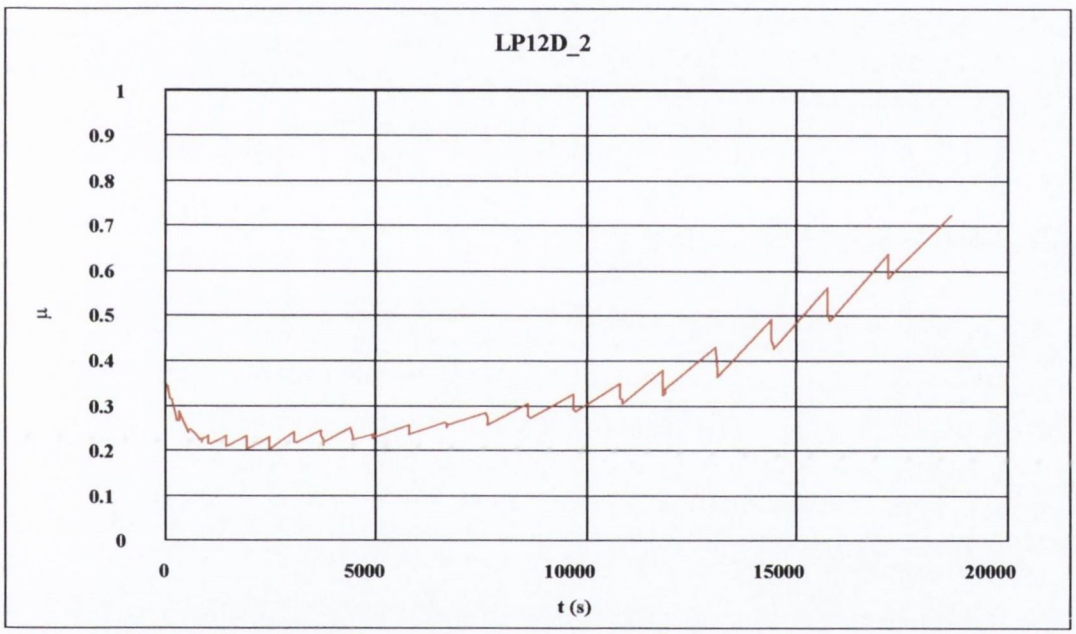


Figure 6.5.2 Lubricity testing of product 12.

Product 12 has a low friction coefficeint for the first 6,900 s (115 min), as seen in Figure 6.5.2 and after that a gradual increase is seen to 9,900 s (165 min), with a more rapid increase occurring thereafter. The friction coefficient of 0.5 is not reached until 15,000 s (250 min), which is a 6-fold increase over the *Carbopol 974P*™ 1% polymer dispersion alone. Product 23 (Figure 6.5.3) has the lowest initial friction coefficeint of the samples examined, with a value as low as approximately 0.18 where it remains until 7,800 s (130

min) approx. Thereafter a gradual increase in the friction coefficient occurs, with it only reaching ≅ 0.3 by the end of the experiment at 18,000 s (300 min).

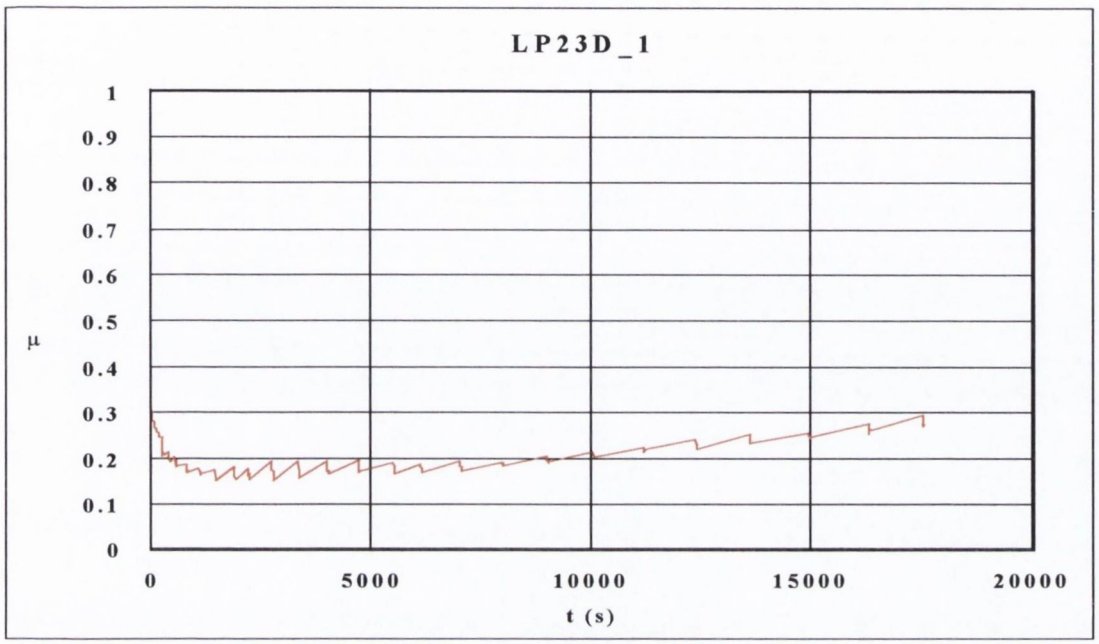


Figure 6.5.3 Lubricity testing of product 23.

The drop in the friction coefficient associated with product 12 compared to *Carbopol 974P* 1% is probably in part due to the reduced apparent viscosity of the sample. However the further reduction in the friction coefficient seen with product 23 cannot be simply explained by a reduction in apparent viscosity as product 23 and product 12 have approximately the same apparent viscosity. The increase in the friction coeffecient over time for the *Carbopol 974P* is most likely due to dehydration of the sample, but this occurs more slowly in product 12 due to the reduced amount of water present and the increased number of excipients which help to retain the water better. Product 23 shows lower friction coefficients than product 12 which is to be expected when an oil is added due to its lubricating nature. What is more interesting is the length of time that the friction coefficeint remains at this low level. The most likely reason for the lack of

increase in the friction coefficeint over time is that the oil forms a barrier to evaporation, preventing the sample from dehydrating to the same level as the two other samples.

6.6 Comparison with commercial products

The leading commercial xerostomia product currently available on the Irish market is *Bioxtra*™. Other products also available include *Luborant*™, *Glandosane*™ and *Mouthkote*™. There are few if any published *in vitro* studies performed on these products. *Luborant*™ has an extremely low viscosity and due to this it was not possible to perform adhesion testing on it. *Glandosane*™ has a low pH and is therefore only suitable for use in edentulous patients. *Mouthkote*™ contains an extract from a plant called 'yerba santa', which is supposed to have lubricating properties, although there are no published studies to support this assertion. Bioxtra™ shows a oscillatory rheological profile similar to *Carbopol 974P*™ 1%, with the crossover between storage and loss modulus occurring at a low frequency, indicating a high level of cross–links and entanglements (Figure 6.6.1). The moduli also show a plateau effect, although not at frequencies as low as seen with *Carbopol 974P*™ 1%.

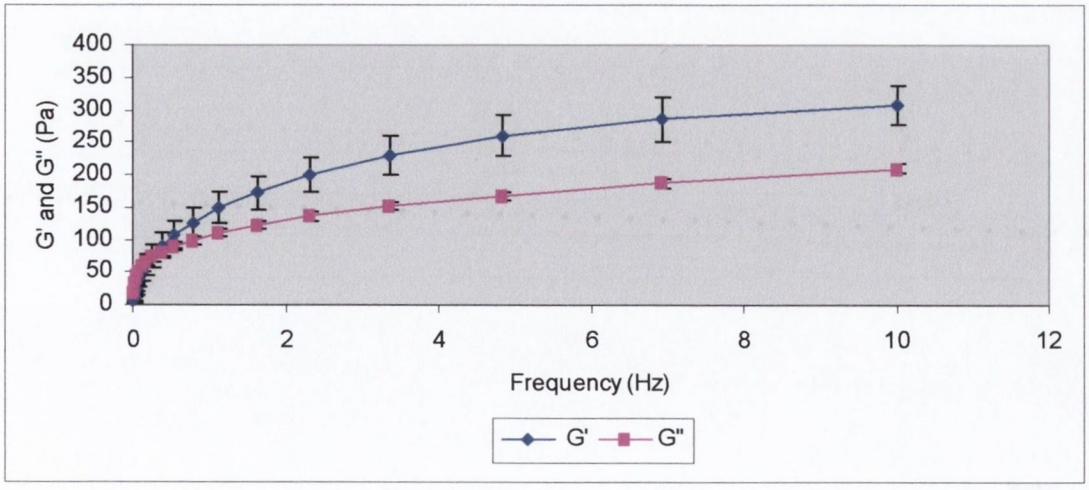


Figure 6.6.1 Storage and loss moduli of *Bioxtra*™.

The bioadhesive profile shows *Bioxtra*™ to have relatively good bioadhesion, similar to that seen with product 12, however it is significantly below that seen with product 23. The bioadhesion also shows a slight drop over time, which is a different profile to that observed with product 23 (Figure 6.6.2).

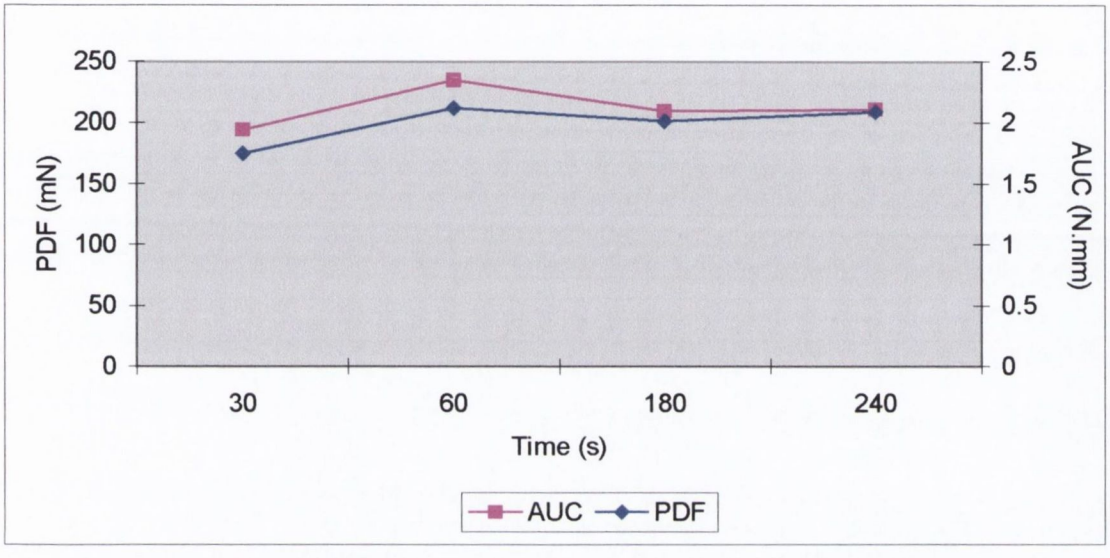


Figure 6.6.2 Bioadhesion of *Bioxtra*™ using FPOM.

6.7 Preliminary *in vivo* trial of xerostomia formulations

Two patients suffering from xerostomia tested product 12 (Table 3.8.1) and product 27 (Table 3.8.3). The only difference between the two products was the presence of 5% sunflower oil, which was present in product 27, but not in product 12. The cause of the xerostomia was different in both cases. One patient was suffering from Sjögrens syndrome. This patient had no unstimulated salivary output, but did have residual stimulated salivary output e.g. when chewing gum. Their chief complaints were difficulty in eating and sleeping, angular cheilitis and oral *Candida* infections. The other person was suffering from drug-induced xerostomia and had residual unstimulated salivary output and stimulated salivary output. The main complaints in this case were polydypsia and difficulty in eating. Neither patient commonly used a saliva substitute.

The patient who had Sjögrens syndrome found that product 12 had an extremely long duration in the oral cavity, in excess of 5 hr, however they found that the product was excessively tacky and gave an unpleasant mouth sensation. On using product 27 the product remained in the mouth for approximately the same length of time. However the patient did feel it was a significant improvement on product 12, with improved lubricity removing much of the unpleasant mouth-feel previously felt with product 12. The patient found the taste of the product pleasant.

The patient suffering from drug-induced xerostomia on using product 27 and product 12 felt alleviation of the symptoms for 75 and 50 min approx for the respective products. However unlike the patient suffering from Sjögrens syndrome, he/she did not feel that product 12 was excessively tacky or that it gave an unpleasant mouth feel. However product 27 was easier to apply and had a longer duration of action. Both products had an acceptable taste, which gave a fresh feel to the mouth after use.

The different opinions each patient had regarding the preferred product is most likely related to the cause of their xerostomia. The patient with Sjögrens syndrome is producing no unstimulated saliva and so the product will remain in the mouth for a prolonged period of time. Due to the length that it is in the oral cavity a certain degree of dehydration of the gel will occur, leading to the product becoming increasingly tacky as time goes by. This will result in an unpleasant sensation in the oral cavity. A second reason for its prolonged residence in the oral cavity is related to the mechanism of bioadhesion that occurs. Due to the fact that there is no mucus covering the oral tissues, mucin/polymer chain entanglement with subsequent van der Waal interactions and hydrogen bonding will not occur. Instead the bioadhesion is most likely to occur through covalent bonding with the mucosal tissue, which forms a very strong bond, resulting in a long retention at the site. The presence of the vegetable oil will prevent dehydration occurring as rapidly and also provides improved lubricity.

The patient suffering from drug-induced xerostomia did not gain alleviation for as long a period as the patient with Sjögrens syndrome. The reasons for this are threefold. Firstly as there is still unstimulated saliva being produced to some extent, the product will be diluted and swallowed more rapidly, secondly as there is still saliva present on the oral tissue this provides a barrier to covalent bonding to occur. Instead the product will create mucin/polymer chain entanglements with subsequent secondary bonding, which while an efficient mechanism of bioadhesion, will not offer the same level of permanence, as the mucus will be replaced more frequently. Thirdly the lemon taste and the presence of citric acid in the product are inclined to stimulate salivary flow, increasing the rate at which the product is lost.

6.8 Conclusions

Historically the development of a successful saliva substitute has been difficult due to the complex nature of saliva, its functions and the problems which arise with the loss of these functions. Xerostomia sufferers can be prone to conditions such as gingivitis, oral *Candida* infection, periodontitis, dental caries and halitosis. A successful saliva substitute should be able to mimic many of the functions of saliva and so address a number of these problems. It should have good bioadhesive properties in order to ensure retention in the oral cavity for a sufficient period of time and be acceptable in taste and consistency. Here a final product, product 27, has been developed that should offer a number of advantages to the xerostomic patient. From a formulation point of view the product developed should help with a number of the problems posed by xerostomia. The product contains salts in levels similar to those found in the oral cavity and so should help buffer the mouth, helping reduce the level of caries. The inclusion of sodium fluoride should also help reduce the incidence of dental caries. Triclosan and sodium lauryl sulphate through their antimicrobial and anti-inflammatory activities respectively will assist in the prevention of gingivitis, *Candida* infection and periodontitis. However without continual use these properties will be lost, so therefore

the taste of the product is important. A fresh lemon flavour was chosen which gives a clean fresh mouth feeling to patients.

The bioadhesion of the product is of considerable significance. If the product cannot be maintained in the mouth for sufficient periods of time, the potential benefits of its use will be lost. Also patients will not be inclined to use a product that has to be replaced at very frequent intervals. *Carbopol 974P*™ was the bioadhesive polymer of choice and extensive *in vitro* testing was performed to determine the bioadhesive properties of the polymer alone and in the formulated products based on *Carbopol 974P*™.

The *Carbopol 974P*™ 1% is predominantly elastic. The oscillatory profile of the *Carbopol 974P*™ 1% provides us with the information that it is highly entangled or cross-linked preventing rearrangement of the molecules (Figure 6.3.1). The apparent viscosity of *Carbopol 974P* is high (Figure 6.3.2) and this will result in the high friction coefficient observed with *Carbopol 974P*™ 1% (Figure 6.5.1).

The oscillatory profile of product 12 and product 23 are different from that of *Carbopol 974P*™ 1%. Product 12 has a storage modulus several times less than *Carbopol 974P*™ 1% as is observed in Table 6.3.1, where values of G' and G'' are given at representative frequencies. The intersection of the storage and loss modulus is at a much higher frequency for product 12 than for *Carbopol 974P*™ 1%, indicating an entangled network as opposed to a cross-linked network (Figure 6.3.5). At lower frequencies product 12 also has a much larger tan delta value than *Carbopol 974P*™ 1%. These major differences in their rheological profiles cause a resultant change in the bioadhesion profiles for product 12, with there being a 41% decrease in bioadhesion compared to *Carbopol 974P*™ 1% when FPOM was used and a 33% decrease in bioadhesion when FPGM was used. A positive synergy was observed with product 12 and mucin (Figure 6.4.2). The apparent viscosity of product 12 is much lower than that of *Carbopol 974P*™ 1% resulting in a significant decrease in the friction coefficient for product 12 (see Figure 4.3.2).

Product 23 shows a slightly lower storage modulus than product 12 with a lower intersection point for G' and G'' (Figure 6.3.5). Product 23 has 26% lower bioadhesion than *Carbopol 974P*™ 1% with FPOM, but has a bioadhesion the same as *Carbopol 974P*™ when using FPGM. This points to the importance of interpenetration and entanglement of mucin chains in this system's bioadhesion, which is confirmed further by the rheological synergism studies (Figure 6.4.3). The presence of the oil would appear also to play a role in preventing overhydration at the mucus/polymer interface and so help reduce the dramatic decrease in bioadhesion over time seen with product 12 using FPGM. The presence of the oil also helps improve the lubricating properties of the product without further reducing the apparent viscosity of the system significantly, and ensures it remains at a low level for a long period of time by helping to prevent dehydration of the sample (Figure 6.5.3).

As previously discussed, xerostomia patients can either suffer from total or partial loss of salivary function. Where there is total loss of salivary function there will be little or no mucus present in the oral cavity. It is therefore important that the product be able to adhere to the oral mucosal tissue. Product 23 showed good bioadhesion with FPOM, having a bioadhesion only 26% less than the *Carbopol 974P*™ 1% alone. This product however also showed good bioadhesion in the presence of FPGM. This would suggest that the product would also be suitable for use in patients suffering from partial loss of salivary function, where there is residual mucus present. Product 23 has bioadhesive properties almost as good as the polymer dispersion alone, however the problem of the tackiness of the polymer dispersion has been reduced with the addition of oil, providing good lubricity to the product.

Chapter 7

Formulation of a bioadhesive fluoride tablet for the oral cavity

7.1 Role of fluoride in caries prevention

Fluoride is important in reducing levels of tooth decay, but its mechanism of action is complex (Section 1.11). In general, fluoride ions are incorporated into and stabilize the apatite crystal of teeth and bone. Deposition of fluoride in tooth enamel increases resistance to acid dissolution and formation of dental caries. Fluoride also promotes remineralization of decalcified enamel and inhibits the cariogenic microbial process in dental plaque.

Fluoride concentrations in plaque are related to concentrations in, and frequency of exposure to the ion in water, foods and dental products. To obtain substantial protection against dental caries, administration of fluoride must be consistent and continuous throughout the period of tooth development and calcification. The most effective means of providing optimal levels of fluoride to large segments of the population is fluoridation of public water supplies; however this has met with controversy in recent times, due to concerns regarding overdosing and related long-term side effects e.g. skeletal fluorosis, fluorosis of the teeth and cancer. Where public water fluoridation is not possible or not allowed, daily administration of individualized fluoride supplements must be used in children to provide adequate levels of protection against dental caries. The use of sodium fluoride supplements would also be useful for protection against dental caries resulting from certain conditions. Patients undergoing chemotherapy and radiation treatment, along with patients suffering from conditions such as Sjögrens syndrome, are highly susceptible to dental caries and could benefit from supplemental fluoride therapy. Fluoride is also used to increase bone density in conditions such as osteoporosis and relieve bone pain in the treatment of various metabolic and neoplastic bone diseases e.g. bone lesions in multiple myeloma.

There are numerous forms of fluoride delivery to the oral cavity e.g. mouthwashes, oral dentifrices and gel trays. However they are not without their problems, which include short duration of activity and difficulty in application. Therefore it was decided to develop a controlled release sodium fluoride tablet that would allow release over a period of at least 8 hr. In order to place it in the oral cavity, a

bioadhesive backing layer was developed, which would allow it to be attached to the oral gingiva.

7.2 Halloysite drug loaded with sodium fluoride

7.2.1 Introduction

Sodium fluoride is a highly water-soluble drug with a solubility of approximately 40 mg/ml. The high water solubility of the drug means that it enters solution rapidly *in vivo*, resulting in a fast release of the drug. In the prevention of dental caries this is not desirable, as fluoride is required to exert its activity locally, over an extended period of time in order to achieve maximum benefit to the dental enamel. Indeed the Martindale (33rd Edition) recommends that oral fluoride tablets currently available, should be sucked or chewed since the topical action of fluoride on enamel and plaque is considered to be more important than its systemic action. As a result of this the development of a sustained release formulation is required, which allows a continuous and uniform release of drug in the oral cavity.

In order to try and provide a sustained release, sodium fluoride was loaded into the excipient, halloysite. Halloysite, as previously discussed (Section 1.6), is a material with a hollow tubular structure, which can help retard the release of drugs over time. However it has been shown to give poor retardation of highly water-soluble drugs, such as diltiazem HCl (Levis, 2000). To try and retard the release of the sodium fluoride further the halloysite was coated using cationic polymers, that bind onto the negative surface charge present. The cationic polymer used was chitosan, which as previously shown can greatly reduce the rate of release of drug from halloysite (Section 4.7.4).

7.2.2 Morphology

The morphology of the halloysite samples double drug loaded with sodium fluoride is similar to that seen with halloysite loaded with tetracycline base and tetracycline HCl. The halloysite drug loaded with sodium fluoride is aggregated into relatively large clumps (Figure 7.2.1), when observed at low magnifications. At higher

magnifications, the halloysite drug loaded with sodium fluoride can be seen to consist of tubules of varying lengths and widths, where typical dimensions are roughly 0.5 μm long by 0.1 μm in diameter. Figure 7.2.2 shows a relatively high proportion of long tubules, which are capable of better drug loading than fragmented short tubules.

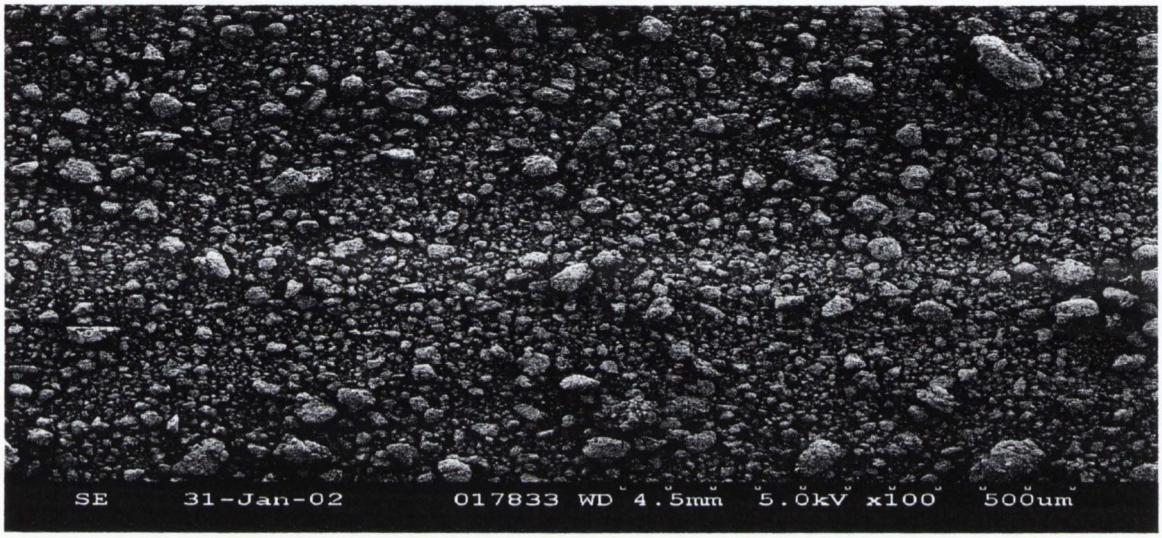


Figure 7.2.1 SEM (X100) of halloysite double drug loaded with sodium fluoride.

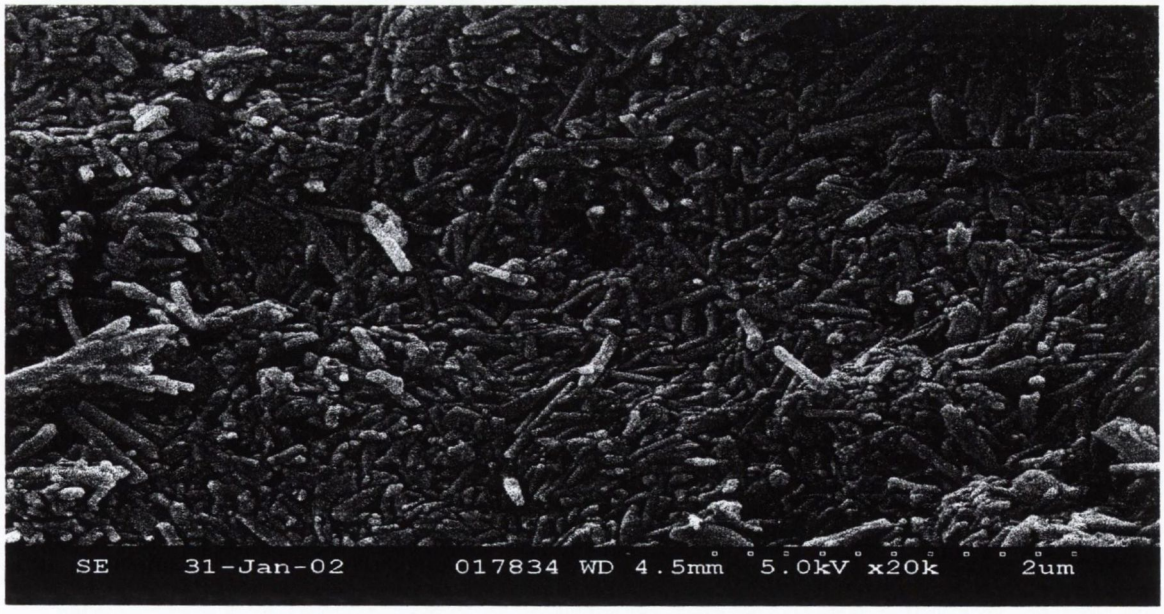


Figure 7.2.2 SEM (X10,000) of halloysite double drug loaded with sodium fluoride.

7.2.3 Dissolution testing of halloysite drug loaded with sodium fluoride

The double drug loading of sodium fluoride (S2) into halloysite gave a 2 fold increase in drug uptake over single loading (S1), as is shown in Figure 7.2.3. This increase in drug loading is not as significant as seen when tetracycline was double loaded into halloysite tubules, where up to a 16 fold increase was seen. This is most likely due to the fact that sodium fluoride does not bind as readily onto the halloysite tubules, with the result that the first loading takes up a significant amount of available intertubular space. This results in the second loading being either equal or less than the first loading, depending on the remaining space available in the halloysite tubules. As the halloysite was able to take another 100% loading of sodium fluoride this would imply that there was possibly more space available to load drug. However the quantities of fluoride required for a therapeutic response are small, ranging from 0.25 mg to 1.0 mg, and therefore it was felt that further loading was not required.

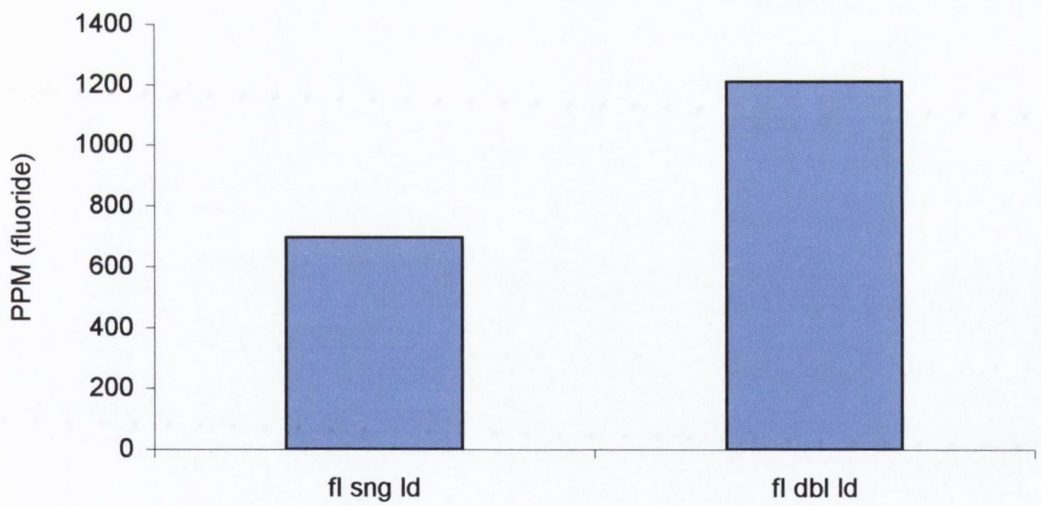


Figure 7.2.3 Increase in drug loading of halloysite with fluoride from single loading (fl sng ld) to double loading (fl dbl ld).

The release profiles of halloysite single loaded with sodium fluoride (S1), double loaded with sodium fluoride (S2), and the control (sodium fluoride) (C1) are shown in Figure 7.2.4. C1 shows 100% release at 200 min, which is a longer release than

would have been expected and is probably due to the sodium fluoride powder clumping in the basket apparatus. A relatively large amount of powder was used in order to ensure detection, and when wetted this formed a solid clump. This resulted in outer layers of the powder going into solution before the inner core of the powder bed was significantly wetted, which significantly reduced the rate of dissolution. The release profiles of S1 and S2 are similar in profile, however S2 shows a higher rate of release than S1, with a larger burst effect. S1 shows 44% release at 380 min, which is a relatively good sustained release, while the S2 system showed 75% release at 480 min. This increased level of release from S2 may reflect a greater amount of intratubular sodium fluoride, which will be released more rapidly than intertubular sodium fluoride (Figure 7.2.3). The release of fluoride from S1 is relatively slow, however there would not be sufficient drug present to provide therapeutic levels. Also while S1 and S2 shows some sustained release of sodium fluoride, it would not be suitable for direct compression into a tablet as halloysite, when tabletted directly, disintegrates rapidly on contact with water. This makes it unsuitable, as the tablet must be able to retain its integrity in the oral cavity over a prolonged period of time.

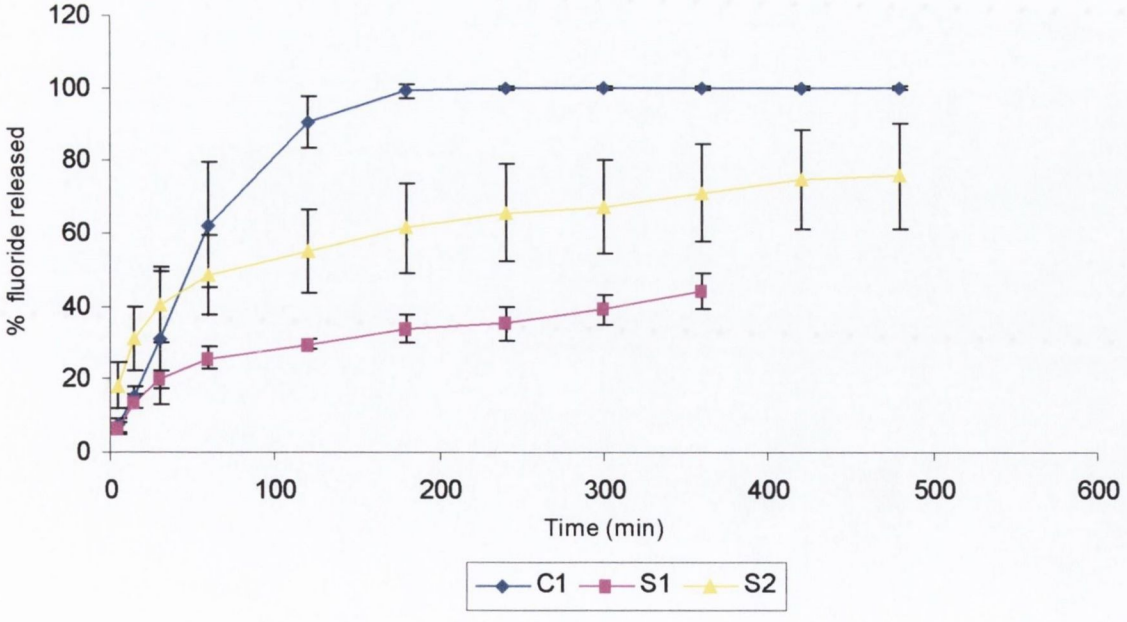


Figure 7.2.4 Release profiles of fluoride.

The effect of a chitosan coating (R1), which binds onto the positive charges on the surface of the drug loaded halloysite, causing reduced release, can be seen in Figure 7.2.5. While the presence of the chitosan coating does not appear to reduce the overall release of the sodium fluoride at 8 hr, it provides a more uniform release, significantly reducing the burst effect observed in the first 60 min. The nature of halloysite with its open end tubules, which allows the drug to leach out, accounts for a large part of the significant burst release seen in the first 60 min. When the chitosan coating is applied it forms a cap over the open end of the tubules. This results in the drug having to follow a much more tortuous path in order to reach the dissolution medium, thus reducing the initial release of drug. There will still be a small burst release due to the presence of intratubular sodium fluoride, which will not be affected by the presence of the chitosan. However at later stages in the dissolution test the rate of release will be greater due to the fact that there is an increased amount of drug still present in the tubules, which will drive the concentration gradient. A certain amount of the drug will be very slow to be released, due to its difficulty in finding a path through the lumen of the halloysite tubules and due to the effect of the chitosan coating. .

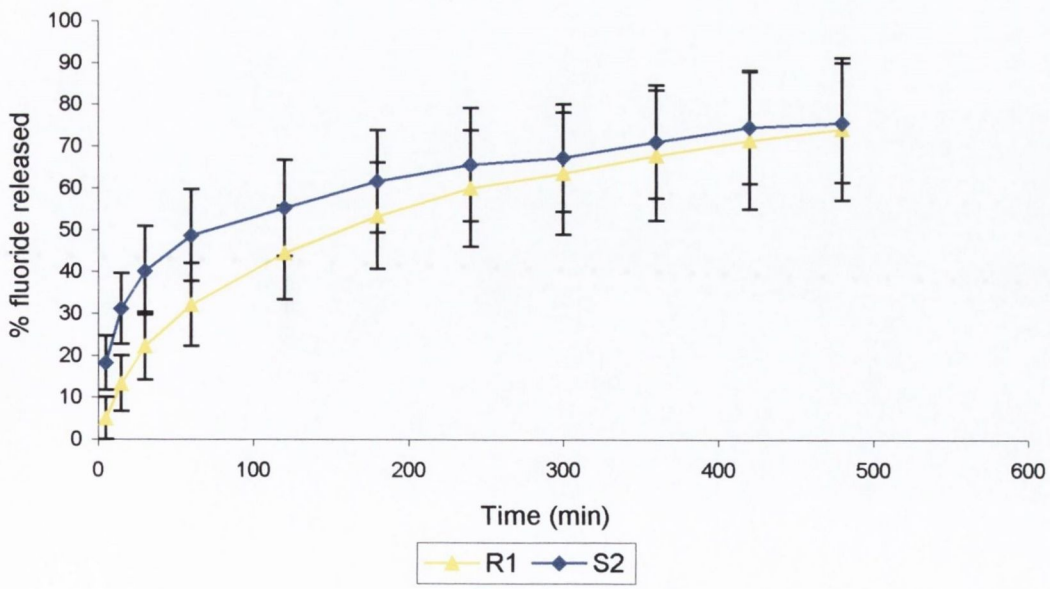


Figure 7.2.5 Effect of chitosan coating on release of fluoride from halloysite.

7.3 Formulation of solid lipid microparticles containing sodium fluoride

7.3.1 Introduction

Sodium fluoride is a highly water soluble drug and therefore it is more difficult to form an effective controlled release delivery system. The use of a solid lipid particle, to further retard drug release was considered to be a suitable complex delivery system as it should help retard drug release significantly. Solid lipid matrices have been long used to provide a prolonged or sustained delivery of drug. The other significant advantage to the use of a solid lipid particle system is that the lipids can be directly compressed and so offer a simple method of formulation, while also providing protection to the halloysite from possible damage that may arise during the tabletting process.

The solid lipid microparticles were prepared by firstly dispersing the halloysite drug loaded with sodium fluoride in a melted lipid, at a ratio of 1:1 halloysite lipid. This was then emulsified, at a concentration of 50%, in a solution containing the following,

Surfactant	5% w/w
Cryoprotectant	5% w/w
Distilled water	to 100%.

This emulsified preparation was then freeze-dried.

Two lipids with different melting points were examined for use in the solid lipid particle, to determine the effect on the release of the drug. The lipids used were *Precirol ATO5*™ and *Compritol 888 ATO*™, both provided by *Gattefosse*. *Precirol ATO5*™ (glyceryl palmitostearate) has a melting point of 53 - 57°C and *Compritol 888 ATO*™ (glyceryl behenate) has a melting point of 69 - 74°C. *Precirol ATO5*™ and *Compritol 888* ATO™ both have regulatory approval for use in pharmaceutical products in Europe and the USA. *Precirol ATO5*™ is approved for use as a lubricant with binding properties and is recommended for lubrication of powders being filled into hard gelatin capsules. It can also be used as a taste-masking agent in wet/melt granulation or hot melt coating. *Compritol 888 ATO*™ is recommended

for use as an inert lubricant with binding properties, as a problem solver in cases of chemical compatibility and for overcoming capping and lamination in compressed tablets. Both *Precirol ATO5*™ and *Compritol 888 ATO*™ can be used as a lipid matrix for controlled release tablets or multiparticulates, in direct compression, wet/ melt granulation and hot melt coating. It is this particular application that is being used in this project. The surfactant used was *Pluronic F68*™ and the cryoprotectant used was glucose. The choice of the surfactant and cryoprotectant were based on work carried out by Levis, (2000).

The lipid systems prepared are outlined in Table 7.3.1. Each lipid system was prepared containing halloysite coated with chitosan using two different binding times of 30 s or 90 s, in order to determine the role of chitosan in retarding drug release in the presence of the lipids. There was a balance to be achieved as the chitosan is coated onto the halloysite by mixing the halloysite in a solution of chitosan in acetate buffer pH 4.2. If the chitosan were not left in contact for a sufficient period of time, full binding would not occur. However if the halloysite were left in the chitosan solution for too long excessive drug could be lost due to its high water solubility. The small amounts of drug present in the halloysite would mean that even a minor loss would be significant to the overall drug release.

Table 7.3.1 Solid lipid microparticle systems prepared.

System	***Abbreviated term***
Halloysite double drug loaded with sodium fluoride (4% w/v), coated with chitosan (30 s), entrapped in *Compritol 888 ATO*™ in a ratio of 50:50, lipid:halloysite product	L1
Halloysite double drug loaded with sodium fluoride (4% w/v), coated with chitosan (90 s), entrapped in *Compritol 888 ATO*™ in a ratio of 50:50, lipid:halloysite product	L2
Halloysite double drug loaded with sodium fluoride (4% w/v), coated with chitosan (30 s), entrapped in *Precirol ATO5*™ in a ratio of 50:50, lipid:halloysite product	L3
Halloysite double drug loaded with sodium fluoride (4% w/v), coated with chitosan (90 s), entrapped in *Precirol ATO5*™ in a ratio of 50:50, lipid:halloysite product	L4
Sodium fluoride entrapped in *Compritol 888 ATO*™ in a ratio of 50:50, lipid:sodium fluoride	L5
Sodium fluoride entrapped in *Precirol ATO5*™ in a ratio of 50:50, lipid:sodium fluoride	L6

7.4 Morphology and dissolution of solid lipid microparticles

The presence of sodium fluoride particles embedded on the surface of the particles of system L5 is clearly visible in Figure 7.4.1 and would probably result in a significant burst release effect when dissolution testing is performed.

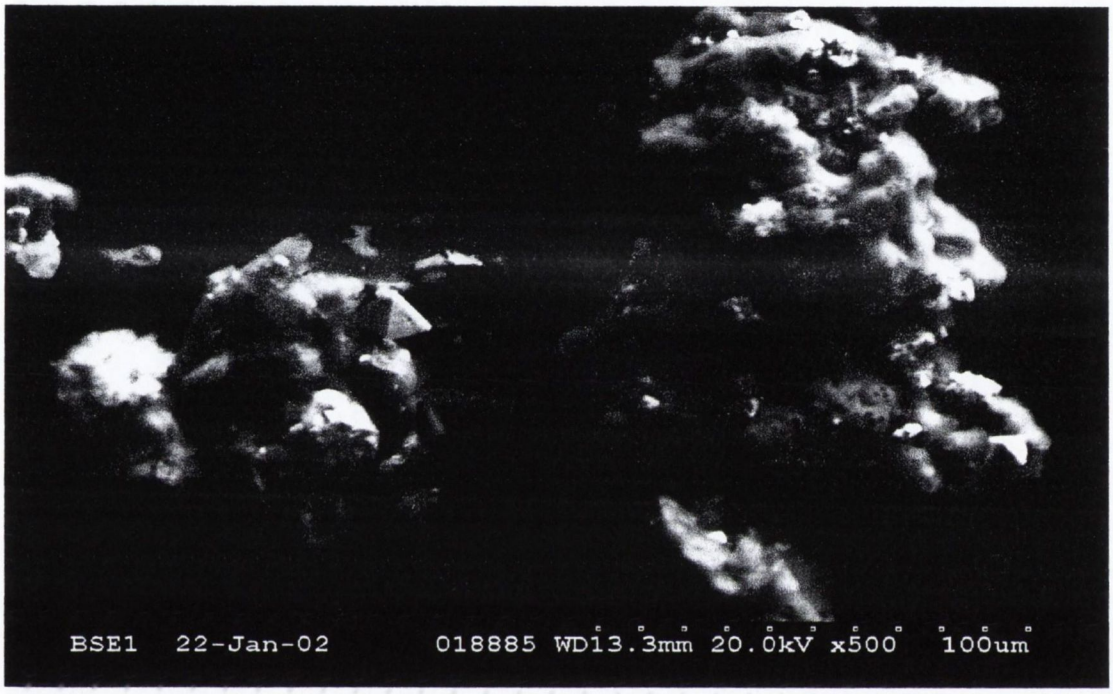


Figure 7.4.1 SEM (X500) of L5.

The halloysite containing (L1, L2, L3 and L4) systems did not give spherical microparticles (Figure 7.4.2 and Figure 7.4.3). The systems L1 (Figure 7.4.2) and L3 (Figure 7.4.3) show the effect of the halloysite on the lipid particles. The particles have a honeycomb appearance as a result of the halloysite tubules, which protrude randomly out of the lipid particles. The presence of the halloysite within the lipid particles provides a double matrix system, through which the drug must travel, prior to reaching the dissolution medium. The drug must first be released from the lumen of the halloysite tubule, and it must then diffuse through the lipid matrix. There are no sodium fluoride particles apparent in the lipid surface of the particles, due to the drug having been loaded into the halloysite tubules prior to formation as lipid particles. This should help reduce any burst release that may occur during dissolution testing.

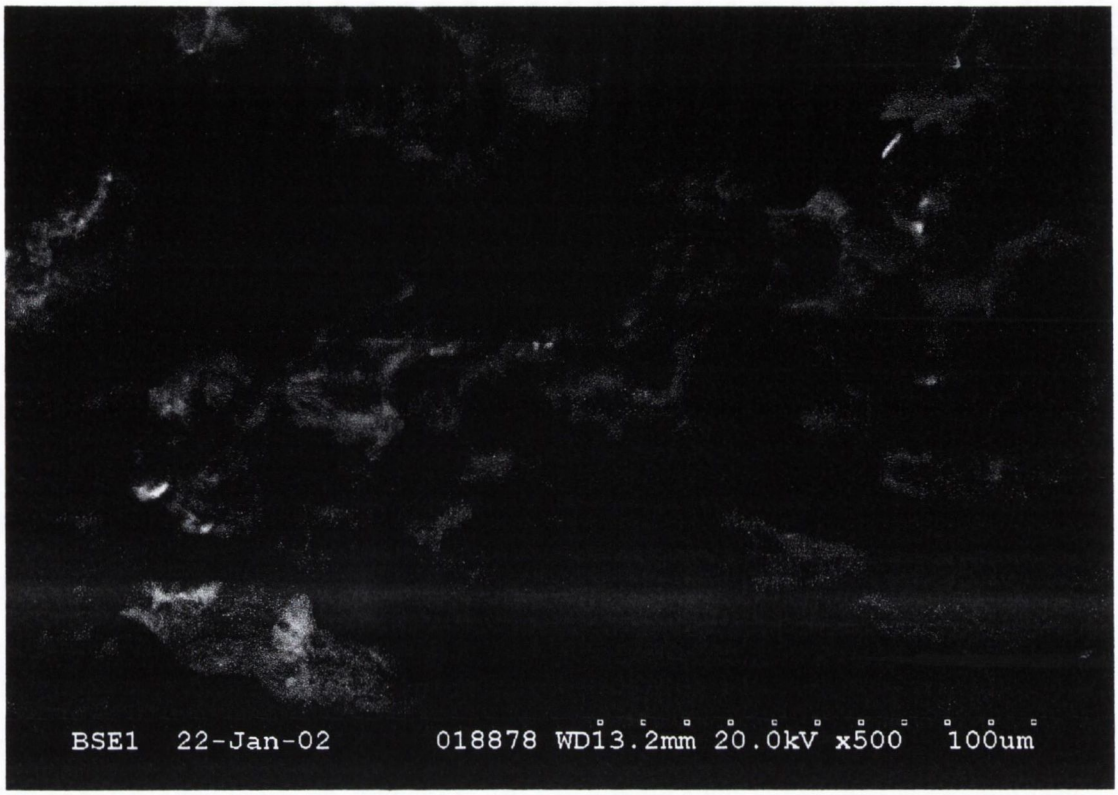


Figure 7.4.2 SEM (X500) of L2.

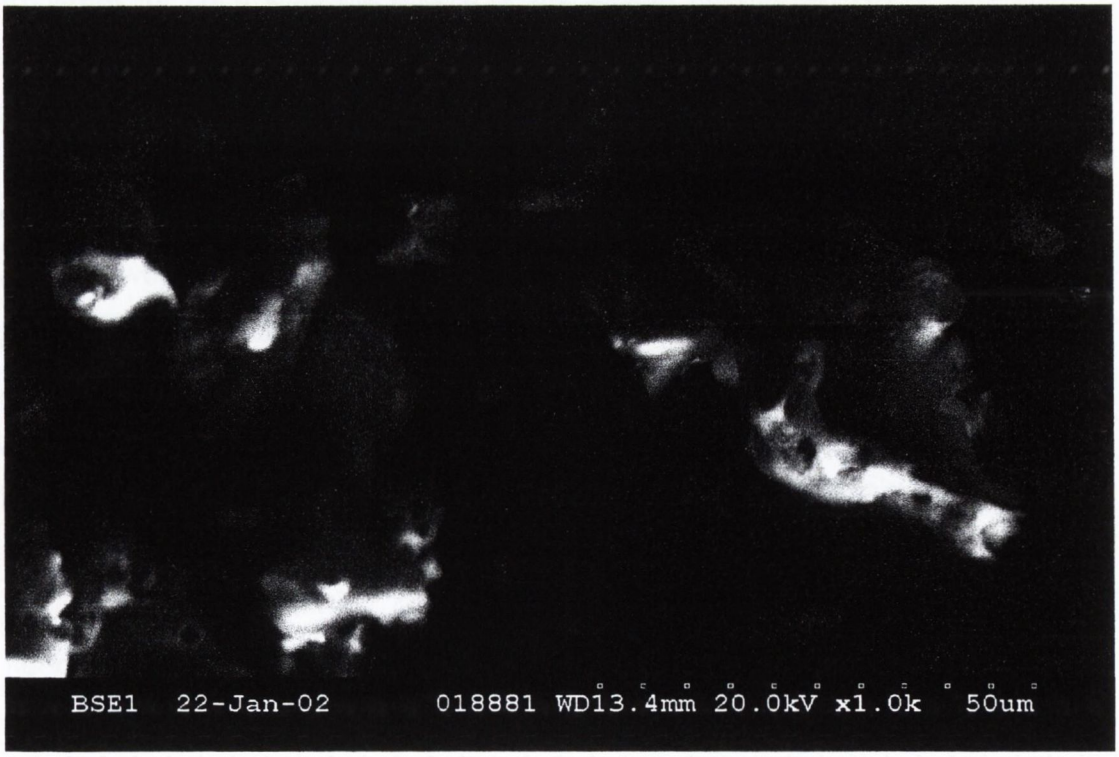


Figure 7.4.3 SEM (X1,000) of L4.

7.4.1 Dissolution testing of solid lipid microparticle systems

The release profiles for systems L2, L3 and L4 are shown in Figure 7.4.4. The fastest release was given by the L2 system followed by L3. L2 gave a gradual release after an initial burst. L4 gave the slowest and most uniform release over time. The results observed here are interesting. *Compritol 888 ATO*™ gives poor sustained release, with L2 showing the same release profile as R1 (Figure 7.4.5). This implies that the presence of the *Compritol 888 ATO*™ offers no advantage in terms of controlling the release of the product. However the presence of *Compritol 888 ATO*™ would be advantageous for the subsequent tabletting process as it is possible to directly compress this lipid.

The presence of the *Precirol ATO5*™ (L4) gives a significant reduction in release over time when compared to R1 (Figure 7.4.5). The difference in release between L3 and L4 also gives an indication of the significance of an increased chitosan binding time, as L3 had a chitosan binding time of 30 s whereas L4 had a chitosan binding time of 90 s as did R1. The *Precirol ATO5*™ would appear to offer good properties for controlling the release of the sodium fluoride as well as having the advantage of being directly compressable, so offering an easy method of production of tablets.

Originally it was thought that the lipid with the lower melting point i.e. *Precirol ATO5*™ would be more likely to suffer deformation of the lipid particles at 37°C, giving a more rapid release than the higher melting point lipid, *Compritol 888 ATO*™. However this is clearly not the case as can be seen from Figure 7.4.4, with *Precirol ATO5*™ lipid particles giving a consistently lower release than those incorporating *Compritol 888 ATO*™. L2 has a $T_{50\%}$ of 180 min while L4 has a $T_{50\%}$ of 480 min. This would imply that the composition of the lipid is of more significance than its melting point in its ability to retard drug release of sodium fluoride.

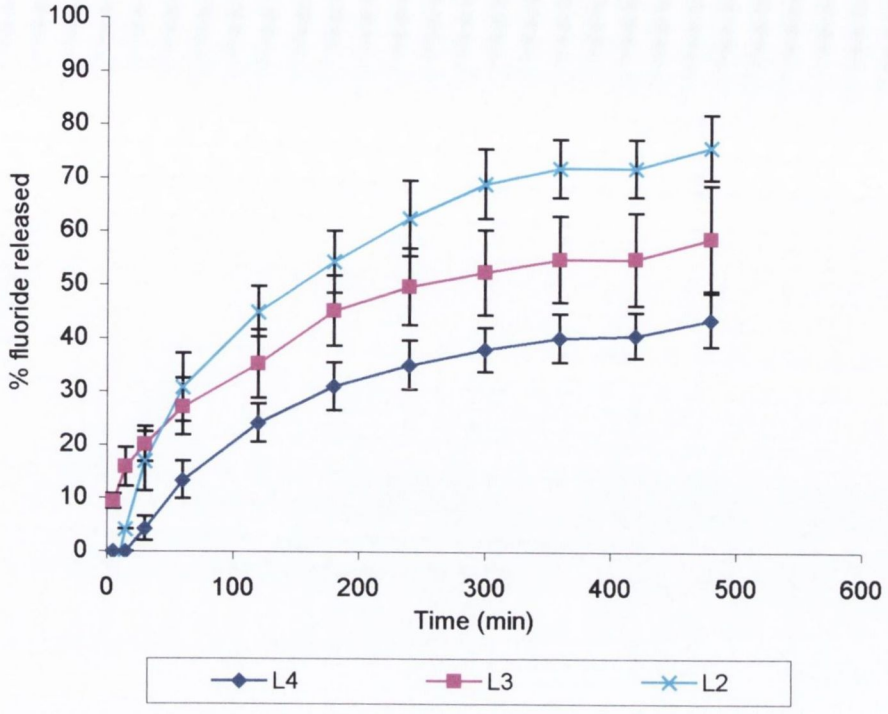


Figure 7.4.4 Release profiles from solid lipid particle systems.

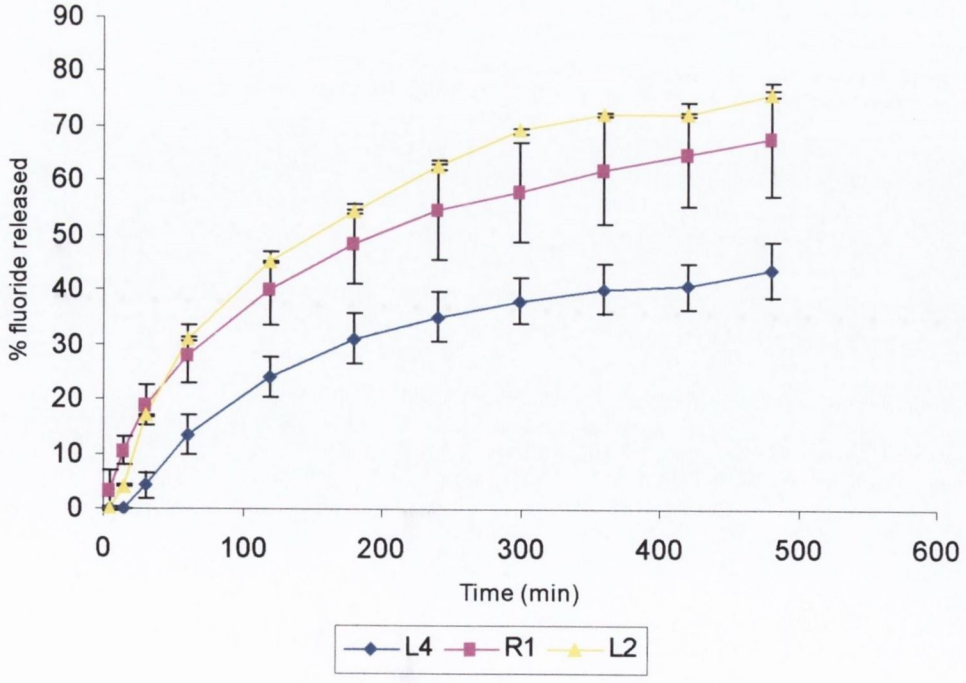


Figure 7.4.5 Release profiles from most favourable systems

The chitosan binding time would appear to be of importance in controlling the release of the sodium fluoride from the halloysite. The binding time of 30 s would not appear to be sufficient to saturate the binding sites on the halloysite. This is reflected in the poor release profile seen with L3 compared to L4. When chitosan binding time was increased to 90 s, a greater level of binding occurred, as shown in the more retarded release observed with L4. The longer binding time would appear to be advantageous with the $T_{50\%}$ values for the *Precirol ATO5*™ systems showing a reduction in release from 240 min at 30 s (L3) binding time, to 480 min at 90 s (L4). It is evident therefore that the inclusion of the chitosan step gives a more effective sustained release and that the binding of chitosan onto the halloysite is an efficient way of retarding drug release.

The systems consisting of sodium fluoride solid lipid microparticles, with no halloysite, showed very poor sustained release (Figure 7.4.6). The system L5 showed a burst effect of 60% release in the first 15 min. This was a faster release than was seen with system C1 (Figure 7.2.4). This may be due to the presence of the lipid preventing wetting and clumping of the product, as occurred with C1, and so allowing a greater surface area for dissolution of the drug. This theory is further supported by the SEM of Figure 7.4.1 where sodium fluoride particles embedded in the surface of the microparticles are clearly seen. However this result is still surprising as the presence of a hydrophobic lipid would be expected to give some retardation.

System L6 does show some controlled release over the time period examined, with a $T_{50\%}$ of 60 min. It would appear that the entrapment of the sodium fluoride was more successful using *Precirol ATO5*™, with an improved controlled release seen using this lipid. The result seen from system L6 correlate well with the result seen from L4 in Figure 7.4.4, which showed an improved control release over R1, indicating a role played by *Precirol ATO5*™ in retarding drug release.

It is apparent therefore that systems L5 and L6 do not offer a good controlled release and that the presence of halloysite in systems L1, L2, L3 and L4 is an important factor in providing a sustained release of the sodium fluoride.

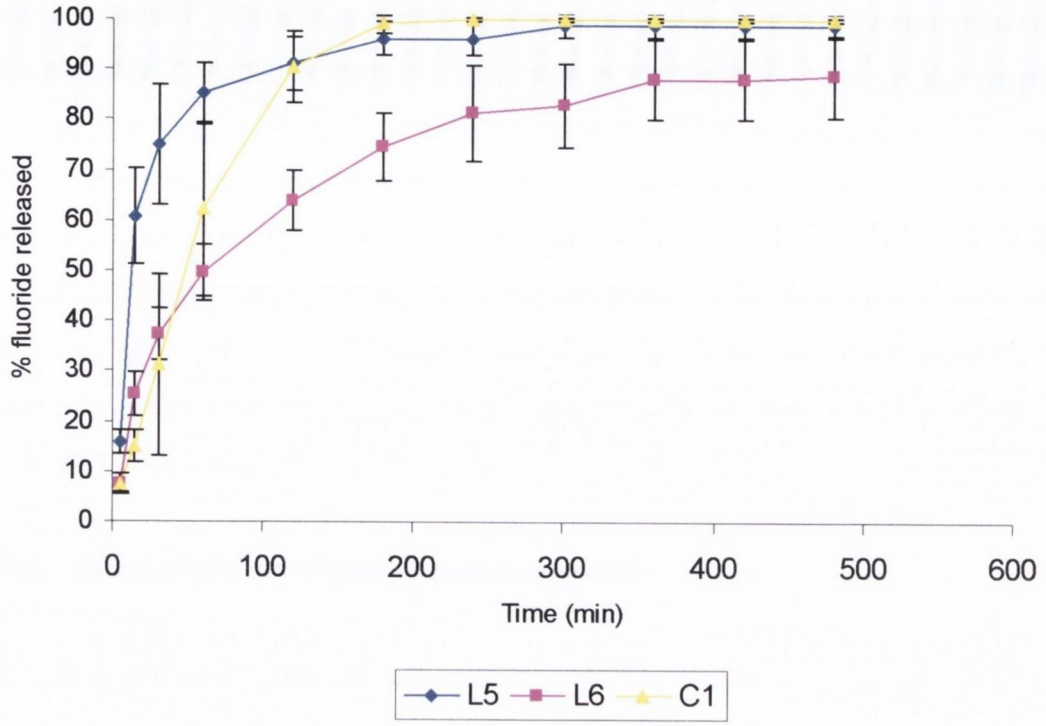


Figure 7.4.6 Release profiles of L5, L6 and C1.

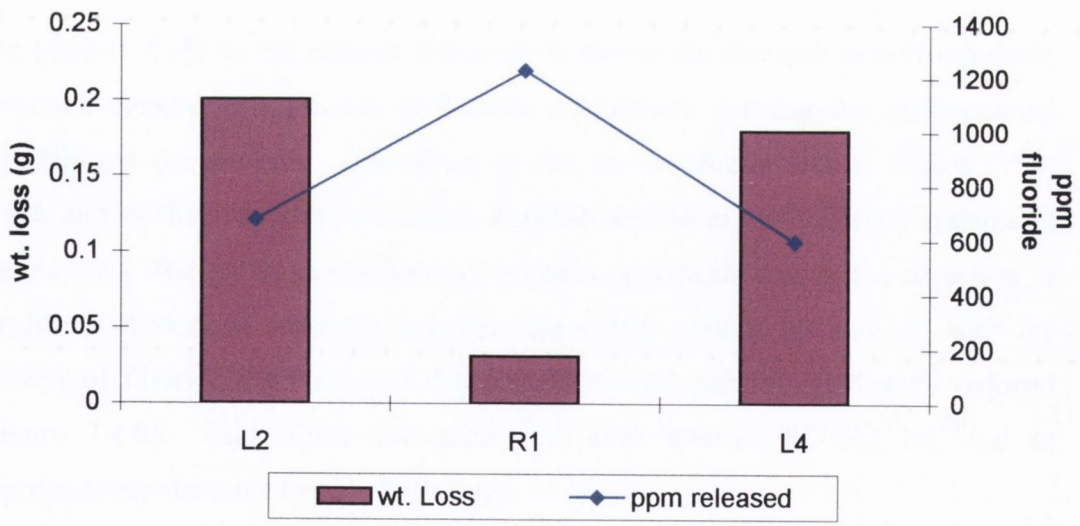


Figure 7.4.7 Weight lost from 500 mg samples of systems L2, L4 and R1 over 8 hr and ppm of fluoride released.

Figure 7.4.7 gives the weight lost from systems L2, L4 and R1 *vs.* the PPM of fluoride released. The chitosan bound halloysite loses the least weight and gives the greatest release of sodium fluoride. The other two systems show far greater weight loss and less fluoride released. The weight loss is due to the presence of the lipid, which means that the sample does not contain as much fluoride. This will mean that larger amounts of the lipid systems will be required in the final formulation, in order to achieve a therapeutic response. However as therapeutic levels are low this should not be a significant issue. In cases where higher levels of drug were required in order to achieve therapeutic levels or where drugs do not have a high potency, it could be a significant problem with this formulation approach.

7.4.2 Effect of pH on release of sodium fluoride

The pH in the oral cavity can vary over a wide range depending on the activities occurring within the oral cavity e.g. after eating carbohydrates the pH in the mouth falls creating an acidic environment. It is therefore necessary to examine the release profile of the systems at both ends of the expected range, to see if this change in pH will affect the release profiles of the systems.

The impact of pH on the release of fluoride is due to the fact that in acid solutions, hydrogen complexes a portion of fluoride in solution, forming the undissociated acid HF and the ion HF_2^-. The effect of this can be clearly seen in Figure 7.4.9, which shows the reduction in sodium fluoride release in the different systems at varying pH. The pH of the buffer used played a significant role in the detection of the levels of fluoride released from the formulated systems L2 and L4, with the amount of fluoride detected over the dissolution test period significantly reduced (Figure 7.4.8). This effect was seen with both systems L2 and L4, and to approximately the same level in both cases.

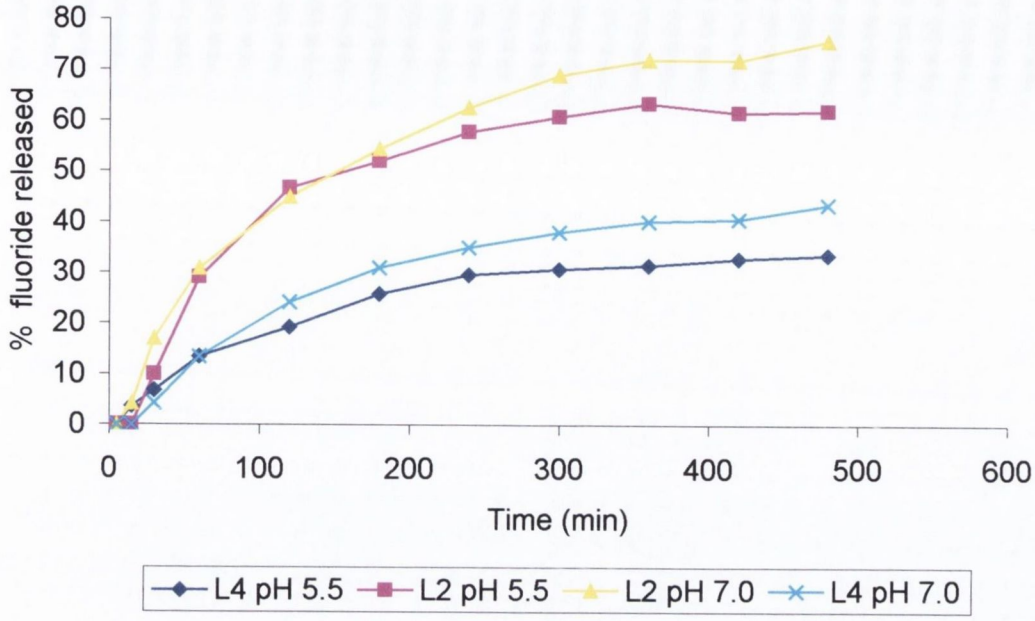


Figure 7.4.8 Effect of pH on the release of fluoride from systems L2 and L4.

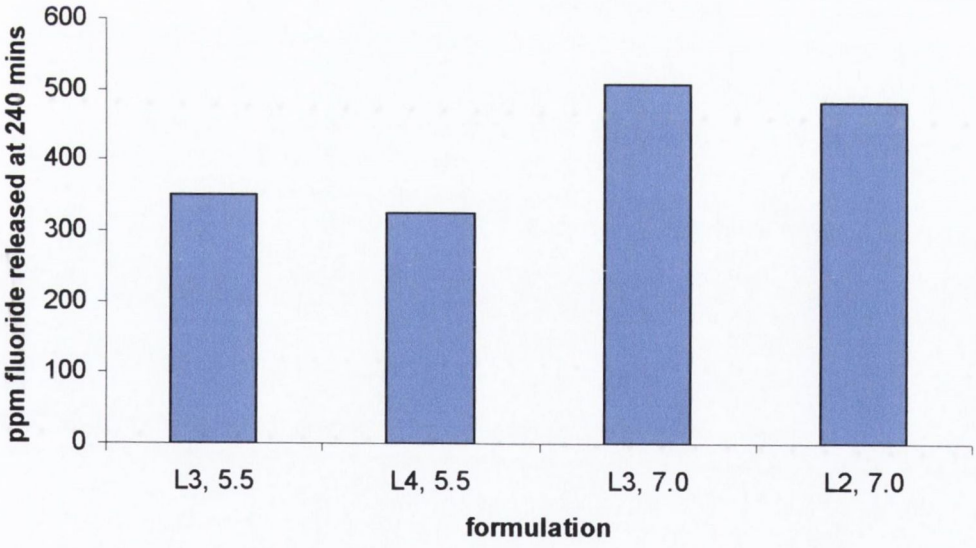


Figure 7.4.9 Complexing effect of pH buffer on fluoride solutions.

This reduction in free sodium fluoride is not a problem in itself; in fact acidified sodium fluoride is taken up to a greater extent into enamel. However where this issue would become a problem is during *in vivo* testing. Fluoride levels are determined from samples using a fluoride electrode. This electrode responds to fluoride ion activity and not to fluoride ions that have been complexed by metal ions e.g. Al^{+3}, Fe^{+3} or to hydrogen fluoride (HF). At low pH, fluoride exists mostly as unionised HF, therefore it is required that samples are at a pH of 6-7 for testing to detect all the fluoride present. The fluoride ion activity also varies with the background ionic concentration and it is necessary that the ionic strength and composition of standards and samples be essentially the same. When saliva samples or dissolution test samples are being taken, if these factors are not taken into consideration a true picture of the concentrations of fluoride present in the saliva will not be achieved. This could potentially result in incorrect dosing of fluoride with resultant side effects (Section 1.11.7). It is for this reason that TISAB is added in equal volumes to all samples taken for testing. TISAB (Total ionic strength adjustment buffer) acts to bring the pH to between 6-7, adjusts the ionic strength of the sample and also contains reagents to release fluorides from complexing metal ions.

7.5 Factorial analysis

Factorial analysis was performed on the percentage drug released at 240 min, for each of the systems L1, L2, L3 and L4 at two different pH buffers pH 7.0 and 5.5 (Table 7.5.1). There were eight dissolution tests performed with 5 replicates for each experiment. It is clear from the data already examined that the different lipids, different chitosan binding times and different buffer pH all have an impact on release profiles. However what is not clear is whether any of these factors interact.

Table 7.5.1 Systems used in factorial analysis and % drug released at 240 min.

System	***pH of buffer***	***% drug released at 240 min.***	***Standard deviation***
L3	7.0	49.75	7.18
L4	7.0	35.08	4.57
L3	5.4	33.87	5.14
L4	5.4	29.66	7.84
L1	7.0	58.33	3.57
L2	7.0	52.55	5.58
L1	5.4	48.23	6.87
L2	5.4	35.52	8.97

Table 7.5.2 Factorial analysis of lipid, pH and chitosan binding times.

Term	***Effect***	***Coef***	***SE Coef***	***T***	***P***
Constant	42.837	1.039	41.22		0.000
lipid	-11.536	-5.768	1.039	-5.55	0.000
chitosan	4.978	2.489	1.039	2.39	0.023
pH	7.077	3.539	1.039	3.41	0.002
lipid*chitosan	-14.374	-7.187	1.039	-6.92	0.073
lipid*pH	3.619	1.809	1.039	1.74	0.091
chitosan*pH	-4.318	-2.159	1.039	-2.08	0.046
lipid*chitosan*pH	-0.957	-0.478	1.039	-0.46	0.648

The results obtained provide us with the following information. All three factors show a main effect. The increase in chitosan binding times provide a significant decrease in the rate of release of sodium fluoride (Figure 7.2.5). The systems containing *Precirol ATO5*™ show a further improvement in retarded release, while the systems containing *Compritol 888 ATO*™ show no improvement over the release seen with the chitosan coating alone (Figure 7.4.4). The lower pH of 5.5

causes a certain proportion of the fluoride to form hydrogen fluoride, which is not detectable using a fluoride electrode (Figure 7.4.8).

However there is also a two factor interaction between chitosan and pH. The lower pH buffer (5.5) causes a reduction in the retarded release offered by the chitosan. This is probably due to the fact that chitosan is soluble at lower pH. As a result at pH 5.5, some of the chitosan bound to the halloysite will go into solution, reducing the retarded release offered by the depleted chitosan film.

7.6 Formulation of a bioadhesive backing layer

7.6.1 Introduction

As well as being able to offer a controlled release of the sodium fluoride over a suitable time period, there must also be a system in place to hold the tablet in the oral cavity. The final system formulated must be able to withstand the numerous challenges that the oral cavity provides e.g. chewing, swallowing, constant saliva production. In order to ensure patient acceptability, it must be an unobtrusive device, comfortable to use and non-irritant. It is also preferable that no special expertise is required for the application of the product. To address these issues it was decided to formulate a bilayered tablet, with a lower layer consisting of a bioadhesive polymer mix.

7.6.2 Bioadhesion

Previous work performed by Codd (1996), showed the *Carbopol*™ polymer to be suitable for use in a bioadhesive backing layer. Therefore this was considered as one possibility. The bioadhesive mechanisms of *Carbopol*™ polymers have been discussed previously in Chapter 6. The other polymer chosen for examination was chitosan, which in recent times has come to notice for its bioadhesive properties (Lehr *et al.*, 1992, Esposito *et al.*, 1996). The chitosan molecules exist in an uncoiled configuration because of positive charges on the repeating units of the polymer backbone. It has been proposed that chitosan acts mainly on the negatively charged sites on the cell surface (Artursson *et al.*, 1994). Drum dried waxy maize

starch (DDWMS) was used as a diluent for the stronger bioadhesive polymers. It shows some bioadhesive properties of its own. The systems considered are outlined in Table 7.6.1.

Sample no.	***System***
1	100% DDWMS
2	95% DDWMS, 5% *Carbopol 974P*™
3	90% DDWMS, 10% *Carbopol 974P*™
4	100% chitosan
5	95% DDWMS, 5% chitosan
6	90% DDWMS, 10% chitosan

Table 7.6.1 Bioadhesive systems for bilayered tablet.

Figure 7.6.1 shows the bioadhesion of the various systems using FPOM. System 3 shows the strongest bioadhesion, however it could possibly be too strong, as it could irritate the mucosal tissue on removal of the system. Systems 2 and 6 showed suitable bioadhesive values and were examined further.

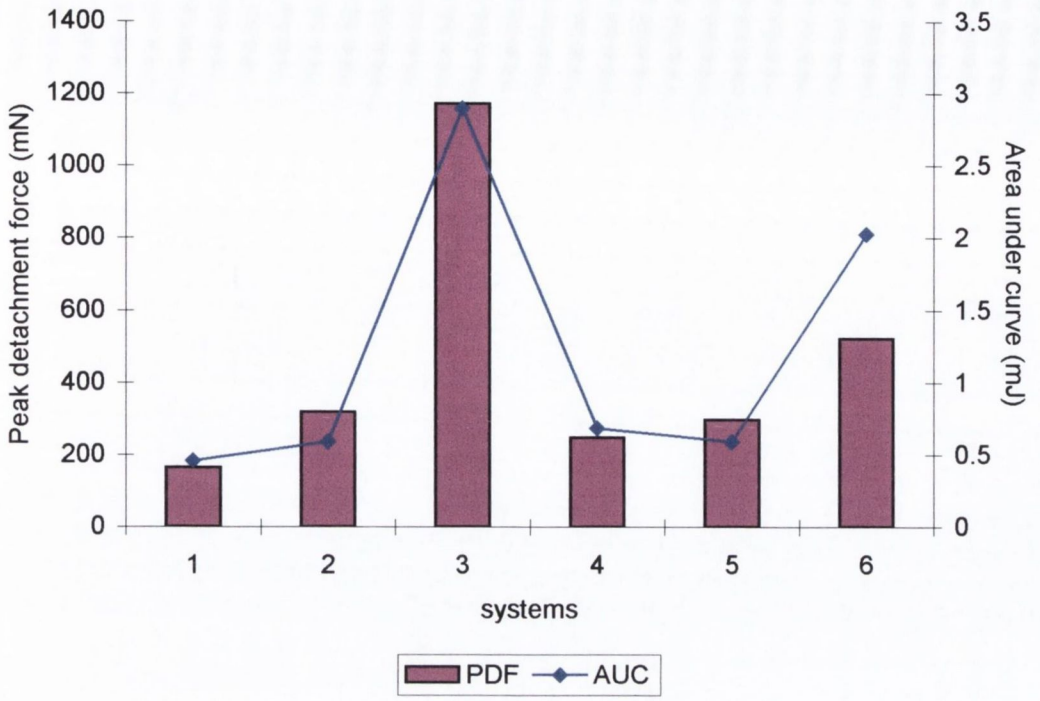


Figure 7.6.1 Bioadhesion of disc systems using FPOM.

The pH of these two systems as well as system 3 was evaluated (Figure 7.6.2). A low pH could possibly cause irritation of the oral mucosa when left in contact over a prolonged period of time. System 5 showed the most favorable pH, being neutral. However in preliminary *in vivo* testing, it was discovered that system 5 disintegrated rapidly in the oral cavity and so was not considered suitable as a bioadhesive backing layer. While the pH of system 2 was significantly lower, Codd (1996), found that it did not cause any mucosal irritation.

There is also a possible advantage to the acidic pH of the bioadhesive layer. Acidification of fluoride solutions increase fluoride uptake by dental enamel. The acidic nature of the bioadhesive layer could possibly create a micro acidic environment, as the sodium fluoride is released and so create an increased uptake into dental enamel.

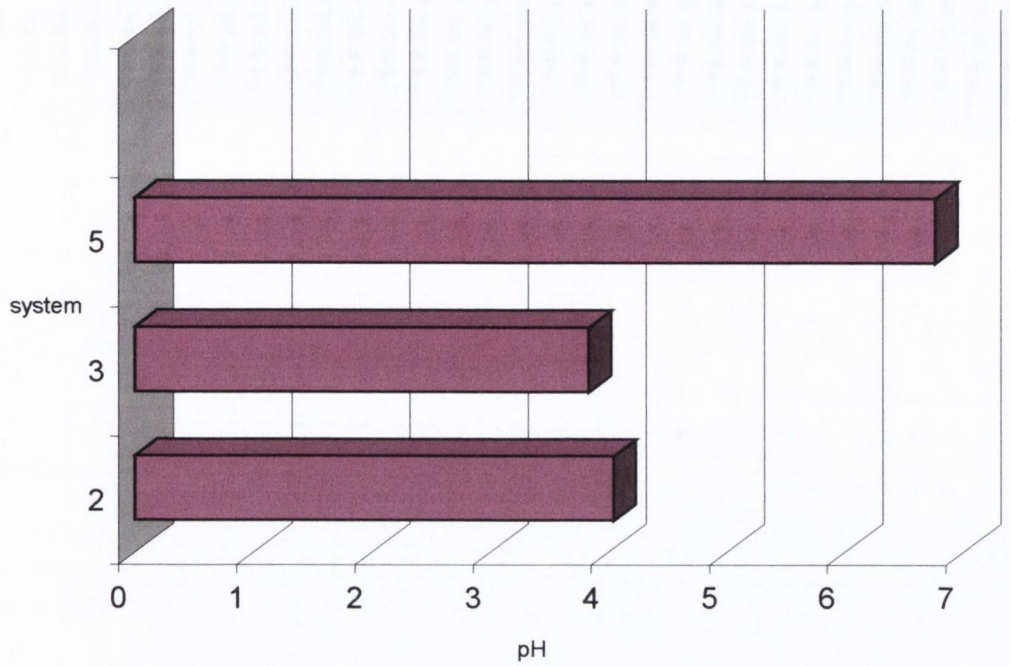


Figure 7.6.2 pH of bioadhesive disc systems.

7.7 Formulation of a bilayered bioadhesive sodium fluoride tablet

Bilayered sodium fluoride tablets consisting of a lower bioadhesive layer (system 2) and an upper layer of either R1, L2 or L4 were prepared. On preliminary examination of these, the tablet containing R1 was found to disintegrate with 1 hr. This is as would be expected, considering the poor tabletting properties of halloysite. As a result this product was disregarded for further studies. However system L2 provided a similar release profile to R1 and was suitable for tabletting, due to the presence of the lipid, *Compritol 888ATO*™. The tablets containing systems L2 and L4 did not disintegrate as rapidly as R1, with disintegration occurring within 3 hr. However this rate of disintegration was still too fast, as the tablets must be able to remain in the oral cavity for a period of 8 hr. In order to try and reduce this premature disintegration, the tablets were annealed for a period of 10 min at temperatures of 62°C and 79°C, for *Precirol ATO5*™ and *Compritol 888 ATO*™ respectively. This had the desired impact of reducing significantly the rate of disintegration of the tablets, with the tablet containing system L2 only showing

disintegration after 5 hr, while the tablet containing system L4 remained intact for the duration of an 8 hr dissolution test.

7.7.1 Tablet hardness

The tablet hardness of the various products is shown in Table 7.6.1. The fact that all 4 systems show almost the same hardness values would indicate that tablet fracture occurred at the bilayer interface. However the hardness values shown are high enough that there should be no concern regarding the tablets prematurely breaking at the bilayered interface during use.

System	*Hardness (Kpa)*
L2	8.42
L2 (annealed)	8.46
L4	8.39
L4 (annealed)	8.4

Table 7.7.1 Tablet hardness of various products.

7.7.2 Dissolution testing of bilayered tablets

The bilayered tablets consisted of 150 mg of system L2 (annealed) or L4 (annealed) and 20 mg of the bioadhesive system 2. The two products showed differing levels of integrity. System L2 (annealed) showed the poorer integrity of the two products examined, with significant disintegration occurring from 5 hr onwards with the discs being completely disintegrated at 7 hr. System L4 did not disintegrate to any significant level during the 8 hr test period, but did form a soft mass.

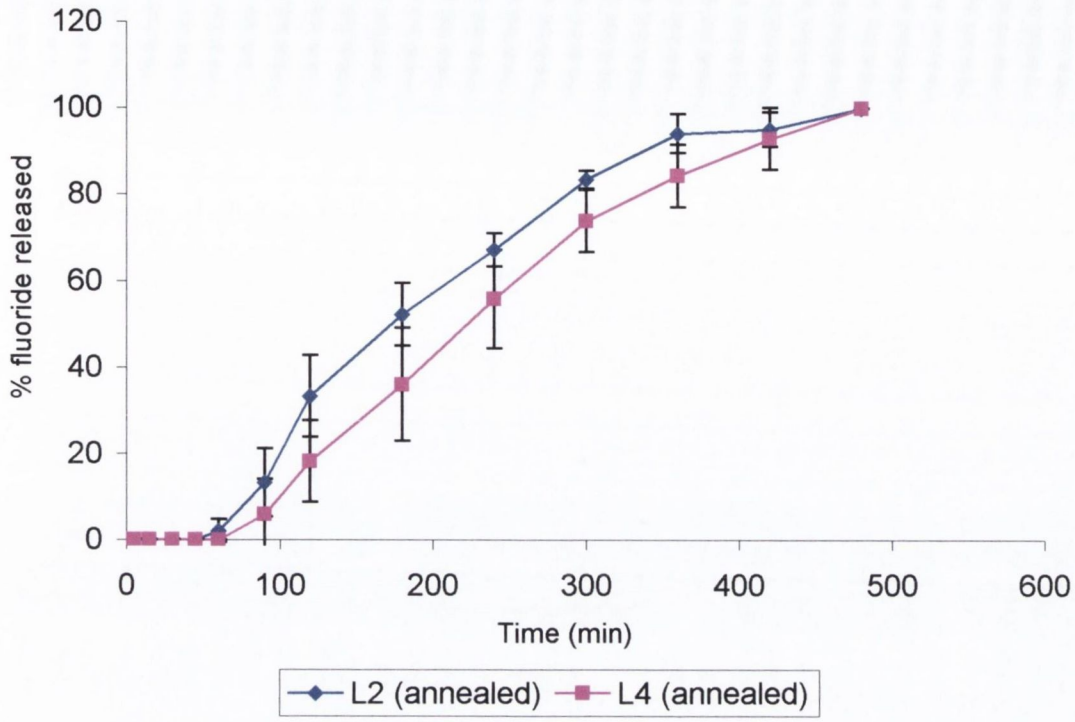


Figure 7.7.1 Release profile from tablets containing L4 (annealed) and L2 (annealed)

The tabletting and annealing process has a significant impact on the release profiles from the solid lipid microparticles. In both systems a decrease in the $T_{50\%}$ value is seen as shown in Table 7.7.2. The $T_{50\%}$ value for L2 is reduced by 60 min upon tabletting and annealing the system, while for L4 it is reduced by 240 min. These increases in the rate of release of sodium fluoride from the lipid microparticle systems are probably due to a number of reasons. The tabletting process could cause a certain amount of deformation to the microparticles, perhaps causing larger amounts of halloysite tubules to protrude through the lipid layer, so causing a more rapid release. Also during the annealing process the lipid is softened and this may result in less lipid coating on the halloysite tubules.

Table 7.7.2 Effect of tabletting and annealing on release.

	L2	***L4***
	$T_{50\%}$	
Microparticles	240	480
Tabletted and annealed	180	240

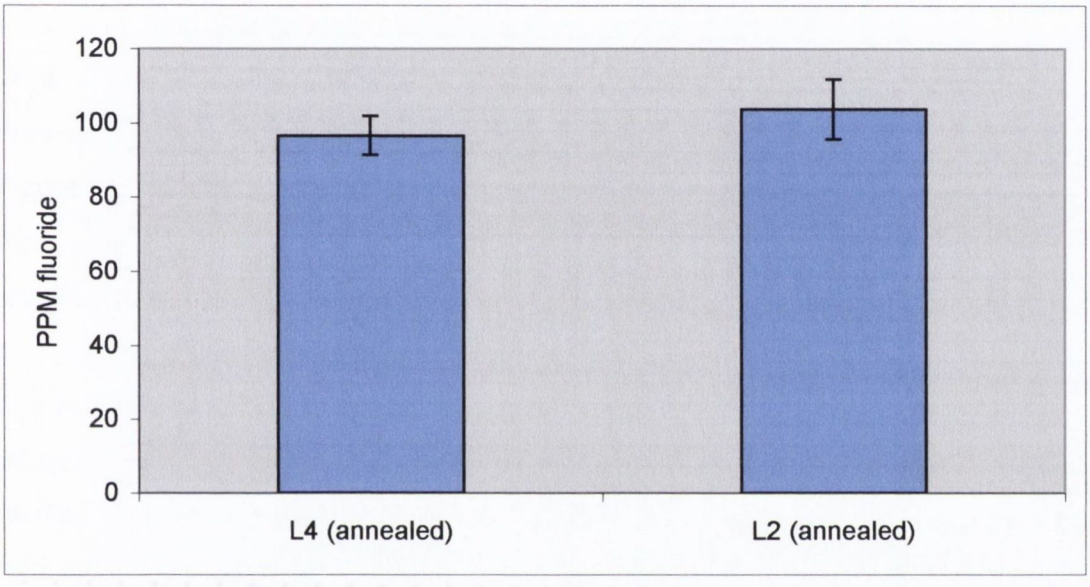


Figure 7.7.2 PPM of fluoride released at 480 min.

The amount of fluoride released in ppm over time as opposed to the % released is important due to the fact that it is necessary to determine whether the amount of system L2 or L4 is likely to give an adequate clinical dose of fluoride to the patient. Both tablets tested showed release of similar amounts of fluoride (100 PPM) at 8 hr, which is an adequate adult dose.

7.7.3 Dissolution testing of bilayered tablets using a flow through dissolution test

It was considered that the Pharmacoepial dissolution test used to determine the release profiles of the tablets in Section 7.6.2 was not sufficiently similar to *in vivo* conditions to reflect an accurate estimation of release. The oral cavity has a salivary

flow varying from 0.3-1.5 ml/min, depending on whether it is unstimulated or stimulated. This would give an approximate release of between 145 ml and 750 ml of saliva over an 8 hr period, with only a small quantity of this ever present at any one time. The Pharmacoepial dissolution test used in Section 7.6.2 used volumes of 1000 ml from the onset. This offers a far greater challenge to the integrity of the tablet than would be given in the oral cavity. Also the amount of turbulence in the dissolution test described in Section 7.6.2 was considerable, with the paddles set at a RPM of 100. This level of turbulence would not be seen in the oral cavity at most times, with maximum turbulence in the oral cavity occurring during eating and drinking, which would not be constant. As a result an in-house flow through dissolution apparatus was prepared as described and shown in Section 3.5.6 and Figure 3.5.1. The lead system, which contained system L4 (annealed) was tested in triplicate, using this in-house method. The release profiles obtained from each sample are seen in Figure 7.7.3.

The flow through dissolution had a flow rate of 1 ml/min, which is an approximate estimation of the flow rate of saliva depending on the activity occurring in the oral cavity. The release of the drug was still slightly greater than was seen with the solid lipid microparticle system prior to tabletting, with release at 480 min being 35.08% for the particle system and 45.23% for the tabletted and annealed system. The use of the flow through dissolution test addressed to some extent the problems associated with the standard Pharmacoepial dissolution test used in Section 7.6.2, like large volumes of buffer and turbulence, and should be more indicative of the release *in vivo*. However, previous studies performed on bioadhesive fluoride tablets *in vivo* showed large amounts of variability (Bottenberg *et al.*, 1991). This variability *in vivo,* was also seen with bioadhesive tablets containing chlorhexidine acetate and miconazole nitrate (Codd and Deasy, 1998).

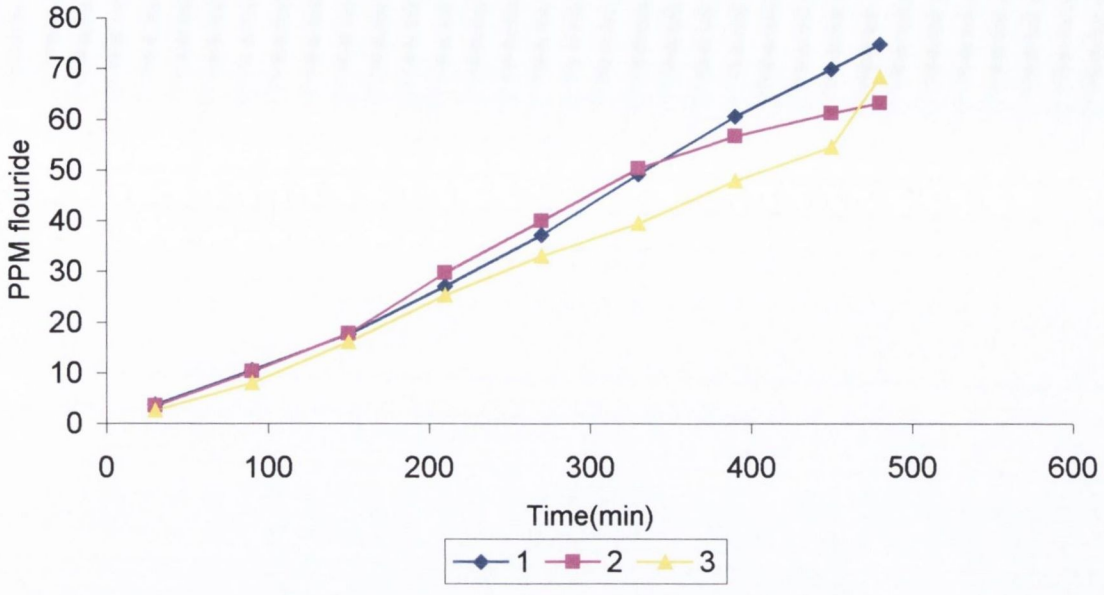


Figure 7.7.3 Release profile of tablet containing system L4 using flow through dissolution test.

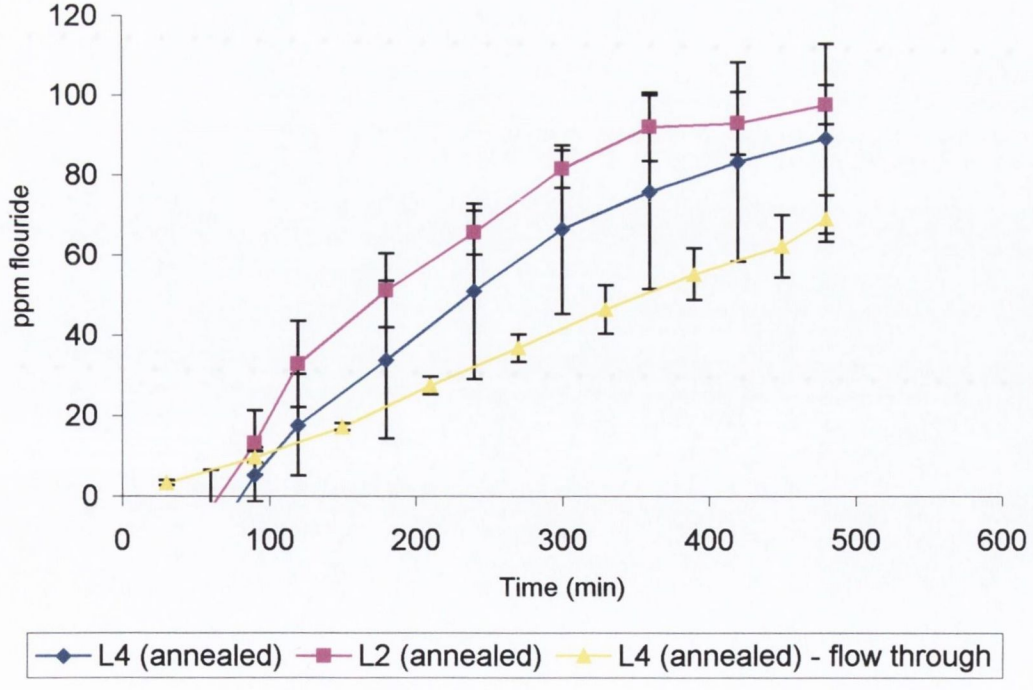


Figure 7.7.4 Comparison of release profiles obtained using Pharmacoepial dissolution test and flow through dissolution test.

The effect of the different dissolution tests on the release of fluoride from system L4 can be observed in Figure 7.7.4 with a significant decrease in the rate of release associated with the flow through dissolution test. This decrease is probably due to the reduced volumes of buffer and the lower levels of turbulence associated with this dissolution test. A final consideration that must be looked at is whether the release of the drug is sufficient to produce therapeutic levels. At 480 min L4 has released 70 ppm approx. of fluoride, using the flow through dissolution test. This is equivalent to 1.1 mg approx. of fluoride. This is sufficient fluoride to treat the 6 – 16 year old range, which require the highest level of fluoride of 1 mg/ daily. The other age groups require lower doses and so the bioadhesive table could be left in place in the oral cavity for a shorter period of time or reformulated to achieve a lower dose.

7.8 Modelling of drug release from systems

Sinclair and Peppas (1984) classify controlled release systems into a number of categories depending on the mechanism of release, with the relevant system here being diffusion controlled devices. Diffusion controlled devices can be either matrix or reservoir systems. Matrix devices have a uniform distribution of drug throughout the device and release of drug from the device is controlled by drug diffusion within the device. Reservoir devices are those in which the drug is found in a central compartment surrounded by a membrane or coat, which is known as the rate controlling membrane.

The rate of release and the duration over which this drug release occurs is important, in order to ensure that drug concentrations remain in the therapeutic region over the desired time interval. A number of models have been developed based on the kinetics of the release. Zero order release is said to occur when the release behaviour of the dosage form is independent of time over long periods (Sinclair and Peppas, 1984). Zero order release is a desirable form of release, as it will result in constant drug concentrations over a prolonged time period.

Another common model used for the examination of sustained release devices is the Higuchi model, which is a diffusional model based on Ficks law. Jalil and Nixon

(1990) state that the Higuchi model for release from a planar surface may be used as a close approximation for the release from irregularly shaped microcapsules. The Higuchi model shows a linear relationship with the square root of time.

Table 7.8.1 Dissolution models fitted to describe sodium fluoride dissolution rate from different microparticulate systems.

Product code	***Zero-order***		***Higuchi***	
	CoDet	**MSC**	**CoDet**	**MSC**
S2	0.708	0.717	0.603	0.744
R1	0.665	0.913	0.979	3.717
L2	0.740	1.166	0.959	3.03
L4	0.826	1.571	0.756	1.229
L4 disc (annealed)	0.970	3.26	0.771	1.224
L2 disc (annealed)	0.920	2.306	0.791	1.346
L4 disc (annealed) flow through dissolution	0.993	4.774	0.766	1.122

Table 7.3.2 illustrates the calculated coefficient of determination (CoDet) values and the model selection criterion (MSC) values when the dissolution data was fitted to the zero order model and the Higuchi model.

The release profile for S2 shows poor correlation for both the zero-order and Higuchi model, most likely due to the significant burst release seen in the first 60 min. When the first 60 min of data are excluded the plot shows strong square root of time dependence with a CoDet of 0.991, making it a good fit for the Higuchi model. When the R1 system is examined, with all the data included, a better fit for the Higuchi model is achieved. This is due to the reduced burst effect seen with the chitosan coating. On formulation of the solid lipid microparticles, system L2 shows

a similar correlation to system R1, with relatively good square root of time dependence. This is as would have been expected as system L2 was shown to have a minimal effect on the release profile of R1 (Figure 7.3.5). System L4 showed poor correlation with the Higuchi model when all the data was included. However when the first 60 min was excluded, due to the lag time observed, a significantly improved CoDet of 0.97 was obtained for the Higuchi model. The presence of the lipid *Precirol AT05*™ is seen to have an obvious impact on the release profile, causing a significant reduction in the release rate, especially over the first 60 min.

When the release profiles from the bioadhesive tablets are examined it is seen that all the systems show a closer approximation to the zero order model than to the Higuchi model. This implies that the tabletting procedure results in a more uniform drug release over time. For both tablets containing systems L2 and L4, the first 60 min of data was not included due to the fact that the fluoride levels were too low for accurate detection. The flow through system used for the tablet containing the system L4 shows the closest approximation to zero order release, with a CoDet of 0.993 and a MSC of 4.774. This improvement over the standard dissolution test is probably due to the increased integrity that the tablet retains over the 8 hr period, which will slow down variable release of the drug.

7.9 Conclusions

The prevention of dental caries is aided by the presence of fluoride. Fluoride is not freely available in the general diet; therefore in order to halt the development of dental caries during tooth formation, fluoride supplementation is required. Dental caries can also be a significant problem where saliva production is reduced or halted, due to the lack of buffering capacity and an increase in cariogenic microflora. This can occur due to drug treatment, radiation therapy or immunological disorders e.g. Sjögrens syndrome.

The best way to ensure fluoride supplementation reaches the widest population of people is to allow the addition of fluoride, in the correct quantities, to the municipal water supply. However there are a number of problems associated with this method. In recent times there has been concerns regarding whether the correct

amounts have been added and the long-term effects of excess fluoride being added e.g. skeletal fluorosis, cancer, chronic fatigue, irritable bowel syndrome, with the result that there has been a call for this practice to be discontinued. Also this method is not applicable in large areas of the developing world, due to the lack of a central water supply for the population. It must also be considered that in certain cases e.g. orthodontic treatment, radiation therapy, extra fluoride supplementation, greater than that found in the normal water supply, may be required.

While there are numerous products which deliver fluoride to the oral cavity, there are drawbacks associated with many of them. Fluoride mouthwashes and oral dentrifices do not have prolonged activity, while gel trays require specialist application and can be messy. The development of a bioadhesive sustained release sodium fluoride tablet was considered to offer a suitable alternative to some of the other methods of application.

A solid lipid microparticle (L4), using the lipid *Precirol ATO5*™, containing halloysite drug loaded with sodium fluoride and coated with chitosan was found to offer the most uniform and consistent release over an 8 hr period (Figure 7.4.5).

A bioadhesive backing layer, consisting of DDWMS (95%) and *Carbopol 974P*™ (5%), offered sufficient bioadhesion for the system to probably remain in place for the required time period (Figure 7.6.1). It also created a micro acidic environment, which would possibly aid in the uptake of sodium fluoride into enamel.

On dissolution of a bilayered tablet containing a bioadhesive backing layer and an upper layer of system L4 (annealed), using a flow through dissolution test, the release profile showed zero order model release characteristics The bioadhesive tablet also showed good hardness and sufficient integrity to last an 8 hr period. The dose of fluoride released over the 8 hr period was sufficient for a daily adult dose. The amount of time the tablet should be left in place could be reduced, depending on the age of the patient.

This bioadhesive tablet would offer a suitable alternative method of fluoride supplementation. In particular it would be beneficial to patients requiring short-

term supplementation e.g. patients undergoing chemotherapy or radiation treatment, or patients with Sjögrens syndrome.

8.1. General discussion

The work of this thesis was to develop three products for use in the oral cavity. The oral cavity can be a difficult area to develop products for due to the numerous problems presented by it. These problems are mainly posed by the constant activity that is occurring within the oral cavity e.g. saliva production, eating, drinking and talking. As a result of these activities ensuring retention of products within the oral cavity can be challenging. In the case of periodontitis, the treatment area is difficult to access as the periodontal pocket is situated below the gum line and is extremely small with depths of 6-13 mm, meaning that only very small quantities of product can be delivered to it. In certain cases the localized application of a formulation may be the only viable option e.g. xerostomia patients with no residual salivary function can only use saliva replacements. Despite these problems there has been considerable interest in the topical treatment of oral conditions, as local delivery of drug possesses many advantages over systemic therapy. These include increased concentrations of drug at the site of delivery while using a far lower dose of drug with a concomitant reduction in side effects (Tonetti *et al.*, 1994, Sakellari *et al.*, 2000). This could be of particular importance in the delivery of fluoride, which is a toxic compound with a wide range of side effects associated with it if overdosing occurs (Section 1.11.7).

Periodontitis is a condition that is characterized clinically by the gum receding away from the tooth, followed by alveolar bone resorption, ultimately resulting in tooth loss. In recent years much research has focused on the microbiological aetiology of periodontitis, with research indicating that certain gram negative bacteria are strongly associated with the condition e.g. *Actinobacillus actinomycetecomitans, Prevotella intermedia, Porphymonas gingivalis* etc (Wong *et al.*, 1999, Hagiwara *et al.*, 1998, Renvert *et al.*, 1996). Antibiotic therapy in periodontitis is based on the premise that specific microorganisms initiate destructive periodontal disease and that the antibiotic agent can exceed concentrations necessary to kill or inhibit the pathogens (American Academy of Periodontology, 1996). Antibiotic treatment of periodontitis is generally reserved for refractory or non-responsive forms of the disease. Until recent times the use of systemic antibiotics was the chosen route

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General discussion

where antibiotics were indicated. However the use of antibiotics systemically for the treatment of periodontitis has been the subject of some controversy due to the variable results seen from clinical trials (Winkel *et al.*, 2001, Lopéz *et al.*, 2000, Winkel *et al.*, 1999). In many cases the results from such trials are difficult to interpret due to variations in study design, small sample sizes, clinically different patient groups and varying regimens. Another problem with these trials is that generally, clinical parameters are examined for improvement after administration of antibiotics e.g. bleeding on probing (BOP), gingival index (GI) and probing pocket depth (PPD), but rarely if ever are the concentrations of antibiotic present in the periodontal pocket determined. Recently a study performed by Sakellari *et al.*, (2000), examined the concentrations of certain antibiotics (tetracycline, doxycycline and minocycline) in gingival crevicular fluid after systemic administration. It was found that concentrations ranged from 0.61 to 1.65 μg/ml after 2 hr and that the range was from 0 to 8 μg/ml, with approximately 50% of samples not achieving levels of 1 μg/ml. These results would suggest that in certain cases individuals may exhibit poor absorption of the antibiotics resulting in poor clinical results, which would also help explain the contradictory results seen in trials using systemic antibiotics for the treatment of periodontitis.

The alternative to systemic antibiotic therapy is the use of local drug delivery systems. In the past twenty years many local drug delivery systems have been formulated and *in vivo* trials performed to examine their impact. Tetracycline fiber therapy (*Actisite™)* has been shown to significantly decrease pocket depth, increase attachment level and decrease bleeding on controlled–force probing compared to scaling (Goodson *et al.*, 1991b). An additive effect of tetracycline fiber therapy when used in conjunction with mechanical debridement has been noted (Newman *et al.*, 1994). However it is difficult to apply, with application taking up to 15 min per tooth. Release is over 10 days and the product is not resorbable and so the patient must revisit their dentist to have the fiber removed. The *Periochip™* is a biodegradable chip containing chlorhexidine. Preliminary data showed that the *Periochip™* when used in conjunction with root scaling and planning gave significant improvement over root scaling and planning alone (Killoy, 1998). In-situ there is an initial peak concentration of 2000 μg/ml chlorhexidine in crevicular

fluid. The concentration of the drug remains above the minimum inhibitory concentration (MIC) for more than 99% of periodontal pocket flora for up to 9 days (Heasman *et al.*, 2001). Ainamo *et al.* (1992) showed that a metronidazole gel (25%) is effective as a monotherapy and attained clinical results similar to that seen with root scaling and planing. When used in conjunction with root scaling and planing, the results seen were also superior to root scaling alone (Griffiths *et al.*, 2000). A week after a single application of a minocycline gel, used as a monotherapy, a significant improvement was noticed in clinical signs with a reduction in bacterial count (Umeda *et al.*, 1996). However when used in conjunction with root scaling and planing no added benefit was seen (Greenstein and Polson, 1998). These gels provide increased drug concentrations for 24 hours after which drug levels decrease rapidly, possibly due to poor retention in the periodontal pockets (Norling *et al.*, 1992).

It was decided to develop a localized drug delivery system that addressed the problems of application and the length of drug delivery. The antibiotic chosen for use in the formulation was tetracycline, as it has been shown to be effective against many of the common periodontopathic bacteria (Miyake *et al.*, 1995, Baker *et al.*, 1985). The controlled release of the drug was considered first. It was necessary to be able to offer concentrations of antibiotic in excess of the MIC of periodontopathic bacteria for periods of time in excess of 14 days. Current formulations, *Actisite*™ and *Periochip*™ offer release over a period of 10 days. *Actisite*™ is the only formulation, which has shown sustained concentrations over the 10-day release period. The release over 10 days in many cases may not be a sufficient time period for complete suppression of periodontopathic bacteria and for this reason a longer release was looked for in this formulation.

In order to achieve this controlled release of drug, the novel excipient halloysite was used. Halloysite has only recently been looked at for use in controlled delivery of drugs, but it has been shown to give effective sustained release of a variety of drugs including tetracycline HCl (Price *et al.*, 2001) and diltiazem HCl (Levis, 2000). It is a microtubular material and it is possible to load drug into the lumen of the halloysite tubules. Halloysite alone does not offer particularly promising release profiles, with significant burst effects and rapid loss of drug occurring. However

when used in conjunction with cationic coating polymers, which are capable of binding onto the anionic sites present on halloysite, a much improved controlled release is observed. Halloysite, which is a natural soil product and is chemically related to kaolin, offers a cheap method of microencapsulation of drug in order to provide controlled release. It is not though, without its problems with the quality of the halloysite varying significantly from site to site, which makes uniformity a problem, in the absence of a purification process. A significant proportion of the halloysite can consist of broken and fragmented tubules, which are of little use for loading drug, with the longer intact tubules carrying the most drug. Therefore samples containing a higher fraction of longer tubules are preferable for pharmaceutical applications. Currently there is no way of separating the broken and fragmented tubules from the complete tubules; therefore the halloysite must be sourced from sites that contain higher proportions of intact tubules.

Tetracycline base was loaded into halloysite using different vessels (freeze-dryer *vs.* dessicator) attached to a vacuum (Edwards RV5) and also different solvents (ethanol *vs.* methanol). When factorial analysis was performed on the loading values achieved, neither variable was seen to have any impact on the final encapsulation efficiency. This is of interest as it shows the drug loading of halloysite to be robust, which is a favourable condition when it comes to large-scale manufacturing. The double loading of halloysite gave an apparent 16-fold increase in encapsulation efficiency, pointing to the importance of subsequent drug loadings. This large increase on the second loading, was probably due to a large proportion of the first loading being bound by the surface charges of halloysite, making it unavailable for release. The release of tetracycline that had been double loaded into halloysite was relatively rapid with a large burst effect and so offered little controlled release. However when the halloysite was coated with the chitosan polymer a more controlled release was seen, with release of tetracycline base occurring up to day 63. This is due to the ability of the cationic polymer to bind to the anionic charges on the surface of the halloysite tubules, forming a coating over them, which retards drug release.

While the release of tetracycline base from the halloysite looked promising, the drug loaded halloysite was not a suitable formulation for direct delivery to the

periodontal pocket. As a result, a delivery system had to be developed in which the drug loaded halloysite could be incorporated. This concept of separating the drug release system and the delivery system is one that has not been examined extensively. In most cases both the drug release and the delivery system have been incorporated into one product e.g. *Actisite*™ fibers both hold the store of drug and are used for the controlled application, with *Periochip*™ the gelatin matrix which comprises the delivery system has the drug incorporated into it, as does the metronidazole dental gel. To separate the two functions during development is an improvement, as each function can be separately optimised prior to their final combination, however they will affect each other in the composite formulation.

The delivery system was required to offer ease of application but must also be robust enough to ensure retention of the product in the pocket for a prolonged period of time (4-6 weeks). A thermoresponsive delivery system was considered to be a suitable option. The polymer *poloxamer 407* was chosen to be the basis of the delivery system. However when its sol-gel transition temperatures were examined at two different concentrations (20% and 25%), it was found that neither concentration offered a suitable transition temperature, with both being below 20°C. It was considered preferable to have the system liquid at room temperature, to ensure ease of administration and therefore it was necessary to manipulate the sol-gel transition temperature. This was done through the addition of PEG, which acts to increase the sol-gel transition temperature (Gilbert *et al.*, 1987). Higher molecular weight PEG's were found to give a sharper transition temperature and so PEG 20,000 was chosen for use. The presence of the PEG however, reduced the overall strength of the system with the storage modulus being reduced by over 40% and therefore there were concerns over retention of the product *in vivo*. In order to try and address this potential problem, the tissue adhesive, octyl cyanoacrylate (octyl CA) was incorporated into the delivery system. Octyl CA is widely used in biomedical applications, e.g. degradable sutures, hip replacements, dental dressings and so was considered a safe option for incorporation into the formulation. Polymerisation of the octyl CA occurs at neutral pH, therefore in order to prevent polymerisation occurring during storage, the pH of the delivery system was 4. This did not affect the thermoresponsivity of the delivery system. On testing the bioadhesion of the final formulation, it was shown to have strong bioadhesive

properties. When the drug loaded halloysite was incorporated into the delivery system, no significant change in the release profile was observed. This would point to the delivery system having no significant effect on release of drug.

There are a number of areas where further research could be carried out on this formulation. Firstly in relation to the choice of antibiotic, a number of areas could be developed. The antibiotic used could be changed for one with better potency e.g. minocycline. As the amount of drug that is loaded into the halloysite is relatively small, better efficacy may be achieved with a more potent antibiotic. Another option would be to incorporate two different antibiotics into the system. Certain bacteria are more susceptible to one antibiotic than another, and where it has been established that there is a mixed infection, particularly where *Actinobacillus actinomycetecomitans* is present, the use of two antibiotics may be preferable. This system would be relatively easy to develop, as the different antibiotics could be incorporated into separate portions of halloysite, which could then be incorporated into the delivery system. Another option would be the incorporation of platelet derived growth factor and insulin-like growth factor into the halloysite, alongside halloysite containing antibiotic. These two factors have been shown to enhance regeneration of periodontal bone and cementum, and could offer a method of further improving the treatment of periodontitis (Lynch *et al.*, 1989). In this way both the bacterial infection and the bone loss associated with periodontitis could be addressed. Finally if a large burst effect was considered to be advantageous, antibiotic could be incorporated directly into the gel, this antibiotic would be released rapidly on application of the product, quickly raising levels above the MIC of periodontopathic bacteria.

The other area of research that would be worthwhile would be the development of an *in vitro* model that correlates with the periodontal pocket *in vivo*. The periodontal pocket is very difficult to imitate *in vitro* due to the small volumes of fluid that pass through it, the extremely small size and the complex nature of the system, with both bone and tissue being present in this micro environment. In this thesis a model was developed that took into account the small volumes present in the pocket, however it did not take into account the possibility of antibiotic binding to tissue. This could be of considerable importance, as it may account for higher

levels *in vivo* for more prolonged periods than would be expected after *in vitro* testing.

In vivo testing of the final formulation developed was performed in dogs using a wound pocket creation model. Each dog had 12 pockets created, with 6 pockets used for the test product and 6 for the control product in each dog. The product was syringed into the pockets and the total weight lost from the syringe recorded. It was calculated that 130 mg approx. was used from each syringe. When the tetracycline content was evaluated this correlated to 130 µg of tetracycline antibiotic being delivered to the pocket. However it is not possible to say that this is the definite amount delivered to each pocket, as there may be considerable loss of product during the delivery process. This is especially true when using the wound pocket model, as the pockets are surgically created, resulting in manipulation of the tissue surrounding the newly formed pockets after the product has been inserted. This problem with determining the total amount of product delivered is a disadvantage and does not occur with other products such as *Actisite*™ and the *Periochip*™. With *Actisite*™ as long as the amount of the fiber inserted is known, the total amount of drug in place can be determined and with the *Periochip*™ the chip contains a specified amount of drug when it is put in place. However this problem would occur with products such as the metronidazole gel, *Elzymol*™.

The pockets were tested at days 14, 28, 42 and 56 for tetracycline content and also for numbers of CFU's under both aerobic and anaerobic conditions. Probing pocket depths (PPD) were also recorded at all sampling time points. It would have been preferable to be able to take more samples over the first 14 days, in order to determine the initial release profile of the tetracycline base. *In vitro* a significant burst effect was observed in the first 3 days and it would be of interest to know did such a burst occur *in vivo*. A burst effect could have a significant impact on the initial reduction of microflora in the pocket and therefore would be possibly advantageous. *Actisite*™ has been shown to release a higher rate of tetracycline for the initial 48 hr after application (Tonetti *et al.*, 1994). However the *in vivo* study did show that tetracycline was present in the pockets at an approximately constant level from days 14 to 42, at concentrations averaging 21.24 µg/100 mg of residue. While this provides us with the information that the product is obviously retained in

the pocket for at least 6 weeks, it is not able to provide accurate information as regards the actual amount of tetracycline in the pocket. This is largely due to the nature of the sampling, which can result in varying amounts of residue being obtained and also the residue can consist of a combination of product, gingival crevicular fluid, blood and saliva. As a result it is not sufficient to indicate that an average release of 21.24 μg/100 mg was released and that this is greater than the MIC of most periodontopathic bacteria.

It was also necessary to examine the microbiology of the pocket to determine if suppression of microorganisms was occurring. This was done by determining the numbers of total CFU's under both aerobic and anaerobic condition. It was shown that the number of both aerobic and anaerobic microorganisms present in the pockets were significantly reduced in the presence of the tetracycline delivery system. However the microbiology performed did highlight a significant problem with this study and that was the fact that the sample used was very small. The importance of sample size was clearly observed when the results from two pockets were seen to skew the entire data set at day 42.

PPD were also recorded over the 6-week period and the test pockets showed a continual improvement over that time period. However the importance of the PPD in this study is questionable as the pockets are surgically created and so could possibly heal differently to a normal periodontal pocket.

The next step with this product would be to perform a clinical study in humans suffering from periodontitis, with full testing of tetracycline concentrations, specific microbiological testing and also testing for clinical parameters e.g. PPD, BOP, gain in clinical attachment. Specific microbiological testing is necessary to determine the product's impact on specific pathogens strongly associated with periodontitis e.g. *P. gingivalis, P. intermedia, A. actinomycetecomitans*. The measurement of CFU's, while giving an indication of the overall reduction of microorganism in the pockets, is not an ideal system, due to the lack of specificity. It was not possible to know, for example, whether concentrations achieved were sufficient to suppress all types of bacteria or whether specific microorganisms were developing resistance over the period examined. Specific bacterial testing was not possible for a number

of reasons, firstly the model used was a healthy dog model and these pathogens may not have been present, and secondly due to the small sample size used the results would have not had statistical significance as counts would have most likely been extremely small.

It would also be necessary to determine whether the longer release profile of the antibiotic from the new product would have a favourable impact on the condition, both in terms of its initial improvement and the relapse rate observed. In order to examine this it would be necessary to perform a trial against another locally delivered product, containing tetracycline, which delivers the drug over a shorter time period.

Xerostomia is more commonly known as 'dry mouth' and is characterized by a reduction or loss in salivary production. Saliva is a complex substance and has a host of functions in the oral cavity. It acts as a moistening agent and a lubricant, which aids in chewing, tasting, swallowing and talking and also helps prevent the oral tissues from dehydrating and cracking. The constant production of saliva helps wash away bacteria and acids from the oral cavity. Saliva has a buffering capacity that is important in preventing the formation of dental caries. It also contains numerous anti-bacterial substances e.g. lysozyme, peroxidase, which act to help prevent bacterial infections occurring in the mouth. With the loss of saliva all of these functions are also lost, resulting in patients with the condition suffering a broad range of complaints. Patients with xerostomia can have difficulty eating, talking and swallowing due to the loss of lubrication given by saliva. The lack of a moistening agent can result in oral tissue dehydrating and ultimately cracking, giving rise to painful fissures and oral ulcers. Patients will also perceive an intense dryness of the oral cavity, resulting in a constant desire to drink. This may give rise to polydypsia and polyuria, with patients finding sleep difficult as they may wake frequently due to the sensation of dryness in their oral cavity. The lack of buffering capacity and antibacterial activity can result in a rapid increase in dental caries and in infections of the oral cavity e.g. *Candida*, gingivitis and periodontitis.

Xerostomia occurs in a number of patient groups. Sjögrens syndrome is a condition characterized by dry mouth and dry eyes, and is an autoimmune condition, often

associated with rheumatoid arthritis (Parke and Buchanan, 1998). Patients receiving head and neck radiation suffer from xerostomia due to the fact that the salivary glands often lie in the line of radiation therapy, causing cellular destruction of the salivary glands (Scully and Epstein, 1996). Drug therapy can also induce xerostomia with a wide range of commonly used drugs causing reductions in salivary output (Korstanje, 1995). Xerostomia has also been associated with diabetes and HIV (Chavez *et al.*, 2000, Schiodt *et al.*, 1992).

Current treatments for xerostomia are either saliva stimulating agents e.g. pilocarpine or a saliva substitute. Pilocarpine, as a salivary stimulant, results in the production of real saliva with all of its inherent advantages, however it is only of use to people with residual salivary activity. It also has a poor side-effect profile and would be contraindicated in a number of patient groups. Saliva substitutes are the only other alternative for patients, however they currently suffer a number of shortcomings. Their duration of activity is too short and they are often tacky in the mouth. This project aimed to develop an improved saliva substitute through addressing independently the issues of most importance and trying to resolve them at a formulation level.

The formulation developed was based on the bioadhesive polymer *Carbopol*™, which has been repeatedly shown to have good bioadhesive properties (Jones *et al.*, 1996). The concentration of *Carbopol*™ used was 1%, which was felt to offer the most suitable consistency while still being able to give adequate bioadhesion. The product was developed from this base gel, with formulation development related to the associated symptoms of xerostomia. Minerals were included in order to help give a buffering capacity to the oral cavity and so help reduce the incidence of dental caries and fluoride was also included for this purpose. Antibacterial compounds, triclosan, sodium lauryl sulphate and zinc were incorporated in order to help increase the oral cavity's ability to fight infection. Sweeteners and flavouring agents were incorporated in order to make the product acceptable in taste. By doing this it is encouraging the patient to use the product on a frequent basis over a prolonged period, which will result in them gaining the maximum benefit from the product. The formulation also incorporated sunflower oil in order to improve the lubricity of the product and so give a more pleasant mouth feel. The presence of the

sunflower oil should also help with activities such as eating, as it will help make the food more slippery as does mucus in saliva.

The bioadhesion of the various formulations was evaluated using a XT-RA texture analyser (Stable-micro systems), in adhesion mode. This texture analyser has been used widely to test bioadhesion, but is only one of a number of methods currently used to evaluate bioadhesion. It is highly subjective, with the results being dependent on a number of factors, such as the probe, the compression force and substrate used. As a result it is important that once a set of parameters has been determined for testing, that these are strictly adhered to. If not the results will be partially dependent on the parameters used and will not represent a true picture of the bioadhesion of the systems being examined. The effect of different substrates was examined during the course of the bioadhesion work, as well as the effect of time and various different formulations. It was found that all three of these factors played a significant role, as well as there being a number of interactions between the systems. It was shown that depending on the type of model used, the mechanism of bioadhesion can change e.g. for FPOM covalent bonding will occur, as this would appear not to be a factor when FPGM is used. With FPGM the dominant mechanism would appear to be interpenetration and entanglement of the mucin chains with the polymer chains, with subsequent secondary chemical bonding. This could be of significance depending on the type of xerostomia the patient suffers, as where there is no residual salivary activity there will be no mucin for entanglement to occur, therefore covalent bonding will probably be the principal mechanism of action, resulting in a stronger bond. However where there is residual activity, salivary mucin will be present. This mucin will firstly prevent covalent bonding from occurring as it will shield the mucosal tissue, and will result in mucin/polymer interpenetration and entanglement with secondary chemical bonding being the principal method of bioadhesion, perhaps resulting in a slightly weaker bioadhesive bond.

The new formulation was seen also to have a significant impact on the bioadhesion observed. The change in conformation of the polymer affected the bioadhesion by affecting the mechanisms of action that occurred in the bioadhesive process. When the rheology of the various formulations was examined these changes in

bioadhesion were more clearly understood. The rheology of the *Carbopol 974P*™ 1% gel (pH 6.75) showed it to have fully expanded structure with extensive cross-linking, which was shown in the high storage modulus and low loss modulus observed. As the polymer is fully expanded, the maximum amount of sites for bonding are exposed on the polymer chains. With FPOM the dominant mechanism of bioadhesion is covalent bonding, which results in strong bioadhesion. However there is a different mechanism of bioadhesion occurring with FPGM, i.e. polymer/mucin chain entanglement and secondary chemical bonding. While there will be extensive secondary bonding occurring on the outer sites of the polymer chains, entanglement will not occur as effectively as it is difficult for mucin chains to penetrate into the fully expanded polymer. The secondary chemical bonding will not give as strong a bond as the covalent bonding, resulting in the lower bioadhesion seen with FPGM. This theory is further supported by the results from rheological synergism studies performed, which showed that *Carbopol 974P*™ 1% gel showed no positive rheological synergy with FPGM, which would be caused by interpenetration of the polymer chains and mucin chains.

The presence of the excipients causes a change in the rheological profile of the system, with a move from a cross-linked system to an entangled system, shown by the crossover at mid-range frequencies of storage and loss moduli. This occurs because the excipients, in particular the minerals and the surfactant, cause the polymer chains to partially recoil, with the result that the chains are no longer in contact with one another, preventing cross-linking from occurring. This results in a drop in bioadhesion, in particular for product 12, due to the fact that the coiled form results in fewer exposed sites for binding, however mucin/polymer chain entanglement should be increased, as there will be more room available between the polymer chains for the mucin to penetrate. It is shown through rheological synergism that entanglement and interpenetration is indeed increased for product 12 and product 23, where positive rheological synergy is observed. Product 23 shows the greatest rheological synergy, which points to strong entanglement. This correlates well with the results from bioadhesion studies, which in all cases showed product 23 to have stronger bioadhesion than product 12.

The lubricity of the final product was considered to be important as if there was not good lubricity the product would feel sticky in the mouth. A number of vegetable oils were incorporated in the product to lower the friction of the product, and there action was evident with friction values being halved by the presence of vegetable oil.

Preliminary *in vivo* trials point to the product having a more prolonged mechanism of action in those patients with little or no residual salivary gland activity. This is probably due to the fact that covalent bonding between the bioadhesive polymer and the proteins on epithelial tissue occurs forming strong covalent bonds, coupled with the fact that there is no saliva being produced which would dilute the product or cause overhydration at the bond interface. Patients with residual salivary activity do not show the same duration of action, due to the fact that the saliva will firstly aid in washing away the product, secondly it will cause overhydration of the bioadhesive bond interface, which has been shown to reduce bioadhesive force and thirdly the mechanism of bioadhesion will be different. The presence of the saliva, will prevent the bioadhesive polymer coming in contact with epithelial tissue and so will prevent covalent bonding from occurring, instead secondary chemical bonding will occur along with interpenetration and entanglement with salivary mucins.

Future work in this area would be primarily to perform a clinical trial in patients with xerostomia. However it would appear important to divide the trial patients into groups based on whether they still have residual salivary capacity or not, as this would appear to be an important factor in determining how effective the formulation is. The effect the formulation has on reducing the incidence of disease in the oral cavity could also be examined. The final area that could be looked at in a clinical trial would be the benefit of the presence of gamma-linoleic acid (GLA) in the formulation. The product could be modified to include greater amounts of oil that is more highly concentrated in GLA e.g. starflower oil, and the effect this may have on saliva production in patients with Sjögrens syndrome could be determined.

Flouride is necessary to help reduce the incidence of caries. Flouride supplementation is most easily achieved by the addition of fluoride to the municipal water supply, which has been shown to be an effective method of reducing the

incidence of caries in children (Baysan *et al.*, 2001, O'Mullane *et al.*, 1988). However in recent times there has been a movement in some countries towards stopping this method of fluoridation due to health concerns. There are also populations who are not served by a municipal water supply and so do not receive this supplemental fluoride. Alongside this group are several patient groups who require additional fluoride supplementation due to increased risk of caries lesions. Among these are patients undergoing radiation treatment, those suffering from Sjögrens syndrome and patients receiving orthodontic treatment. Caries prevention in subjects with radiation-induced xerostomia is achieved by the aggressive and daily use of supplemental fluorides. In the absence of such fluoride Dreizen *et al.* (1977) found that subjects developed a mean of more than 2.5 carious surfaces per month.

Current treatments available are numerous and in many cases successful (section 1.11.6), however there is currently a gap on the market for a product, which can be easily applied by the patient on a regular basis and which will offer a continuous slow release of fluoride over an extended time period. Therefore it was decided to develop a bioadhesive sodium fluoride tablet, which would provide release over at least an 8 hr period. Halloysite was used to provide a controlled release of sodium fluoride, however due to the high water solubility of sodium fluoride, this alone did not provide sufficient retardation of drug release. On addition of a chitosan coating there was a significant reduction in the burst release occurring in the first 60 min, with a more uniform release being observed over the 8 hr period. While the controlled release was adequate the system was unsuitable for tabletting, as halloysite disintegrates partially on tabletting. Therefore it was decided to formulate a solid lipid microparticle, using two different lipids, *Precirol ATO5*™ and *Compritol 888 ATO*™. The Precirol ATO5™ was observed to give an improved controlled release over time, whereas the Compritol 888 ATO™ gave no further retardation of drug release over what was seen with the chitosan coating. A problem associated with the lipid coating is that a large proportion of the system is composed of the lipid, resulting in a lower amount of drug available, however only 1 mg approx. of fluoride is required for a therapeutic response to be achieved.

A bioadhesive backing layer was developed which contained 95% DDWMS, 5% *Carbopol 974P*™ and this showed good bioadhesion properties *in vitro*. A bilayered tablet, consisting of an upper layer of a sodium fluoride containing system (L2 or L4) and a lower layer of the bioadhesive polymer mix was tabletted, using a Manesty hand tablet press. On placing these tablets into a liquid medium, they showed significant disintegration over a 1 hr period. This was unsuitable as the tablets must be able to retain their integrity over at least an 8 hr period. To reduce the disintegration occurring the tablets were annealed, with the system containing *Precirol ATO5*™ (L4) being annealed at a temperature of 62°C and the system containing *Compritol 888 ATO*™ being annealed at 79°C. This slowed down the rate of disintegration markedly for the tablet containing L4, which remained intact over an 8 hr dissolution test. The tablet containing system L2 showed improved integrity, but did start to disintegrate at 5 hr. The tabletting process was also seen to cause an increase in the release rate of sodium fluoride, with $T_{50\%}$ being reduced by 1 hr for the tablet containing the L2 system and by 4 hr for the system containing the L4 system. However there was no burst effect and the release was uniform over the time period examined. The Pharmacoepial dissolution test used was not an accurate simulation of *in vivo* conditions, due to the high volumes of buffer used and the excess turbulence in the system. The oral cavity has an average salivary flow rate of 1 ml/min approx., with only a small volume of fluid ever being present in the oral cavity at any given time. Therefore in an effort to mirror these conditions more accurately, a flow-through dissolution apparatus was developed. This showed a lower and more uniform release of drug over the 8 hr period, with a total drug release of 1.1 mg, which is a suitable dose for a 6-16 yr old.

The final product developed would be easy to apply and should offer a controlled release of fluoride over an 8 hr period. This product would be most suitable for patients who require supplemental fluoride over a short time period e.g. radiation patients, orthodontic patients, but it could also be used in area where there is low fluoride levels in the water. *In vivo* trials would need to be performed in order to determine the exact nature of the *in vivo* release as well as the bioadhesiveness and integrity of the tablet.

Overall three formulations were developed and in all cases they showed advantages over products currently available on the market for similar conditions. The product for the treatment of periodontitis showed improved ease of administration and retention over many of the commercial products available. It also showed a more prolonged release of drug for up to 6 weeks. Further studies would need to be performed in humans, to determine the full usefulness of this novel drug delivery system. The formulation developed for the symptomatic treatment of xerostomia, was a complex product that offers numerous benefits to the xerostomic patient. *In vitro* it showed good bioadhesion and low friction values, while *in vivo* it appeared to be of most benefit to a patient suffering from more severe xerostomia. The product would need to be examined *in vivo,* over a longer time period, to determine the extent to which patients would benefit from it. The bioadhesive fluoride tablet gave a uniform release over an extended time period, which would provide adequate concentrations of fluoride to the oral cavity. It was easy to apply and should cause no irritation *in vivo*. However *in vivo* testing is required to determine the nature of the release in the oral cavity. This product could have a wide treatment group, especially where municipal water supplies are not fluoridated.

Collectively the three novel products developed appear superior to existing products in their treatment classes. As such they represent an innovative approach to the improved treatment of many common disorders that affect the oral cavity. Further refinement as necessary and supportive clinical trials should hopefully confirm their collective contribution to the development of improved drug delivery systems for the successful and convenient treatment of many diseases of the mouth, and may have applications in other regions of the body.

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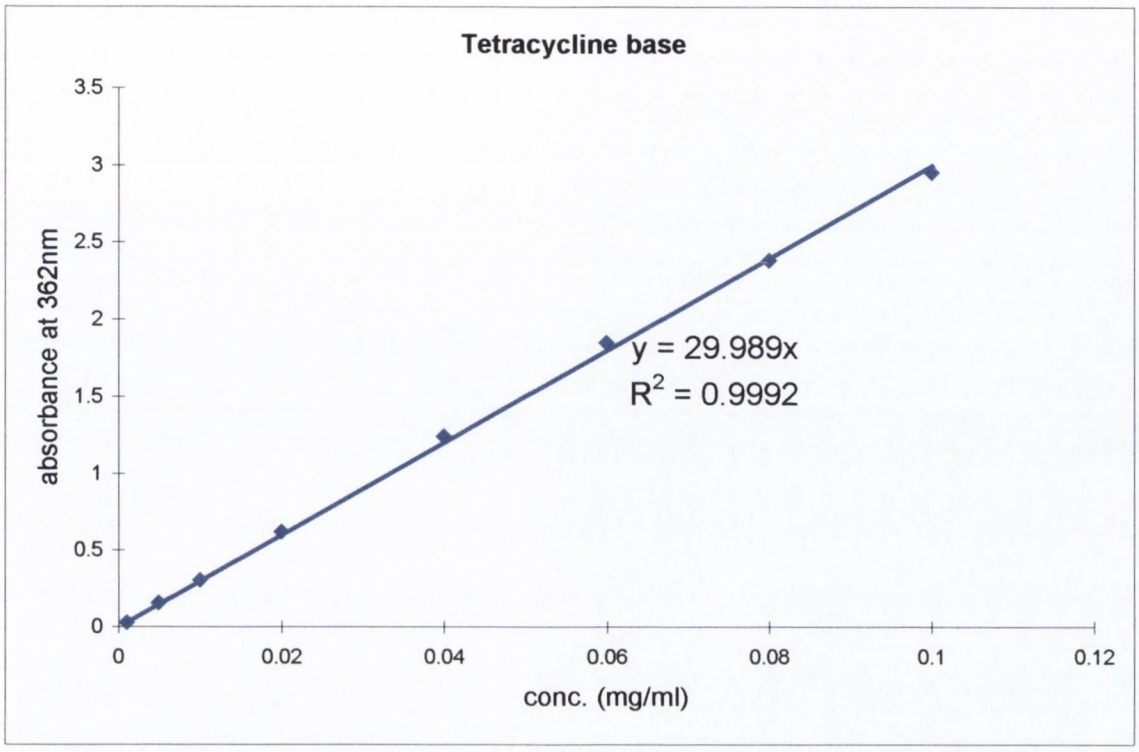
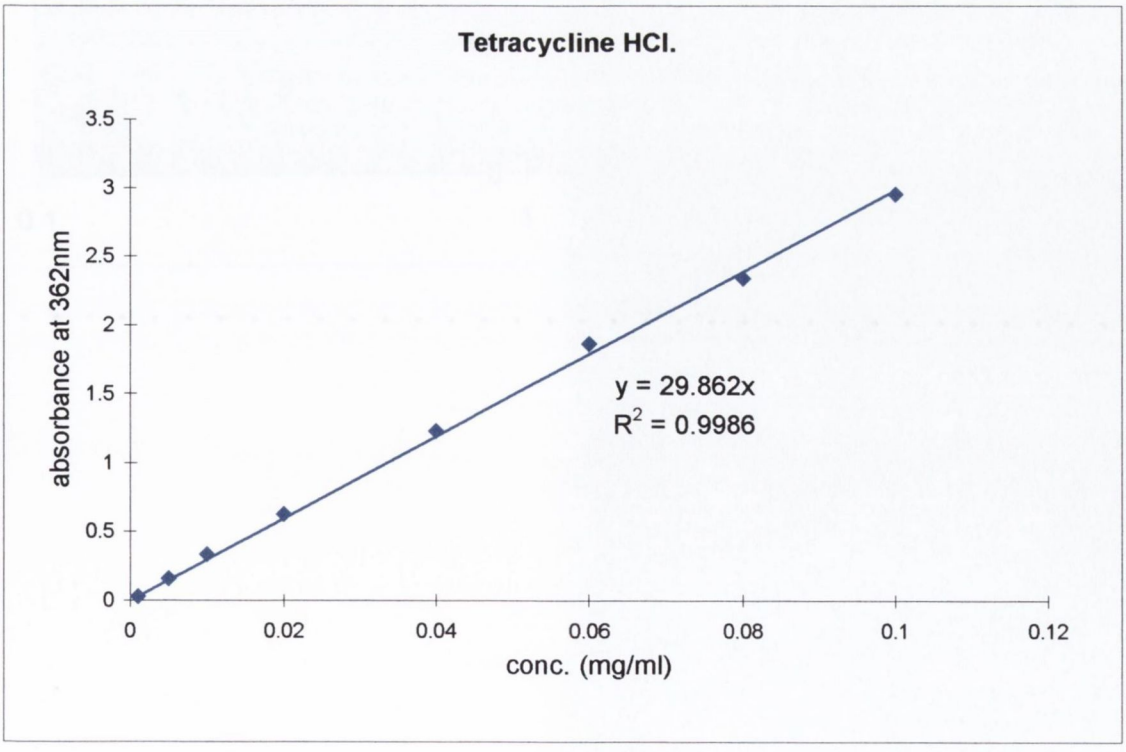
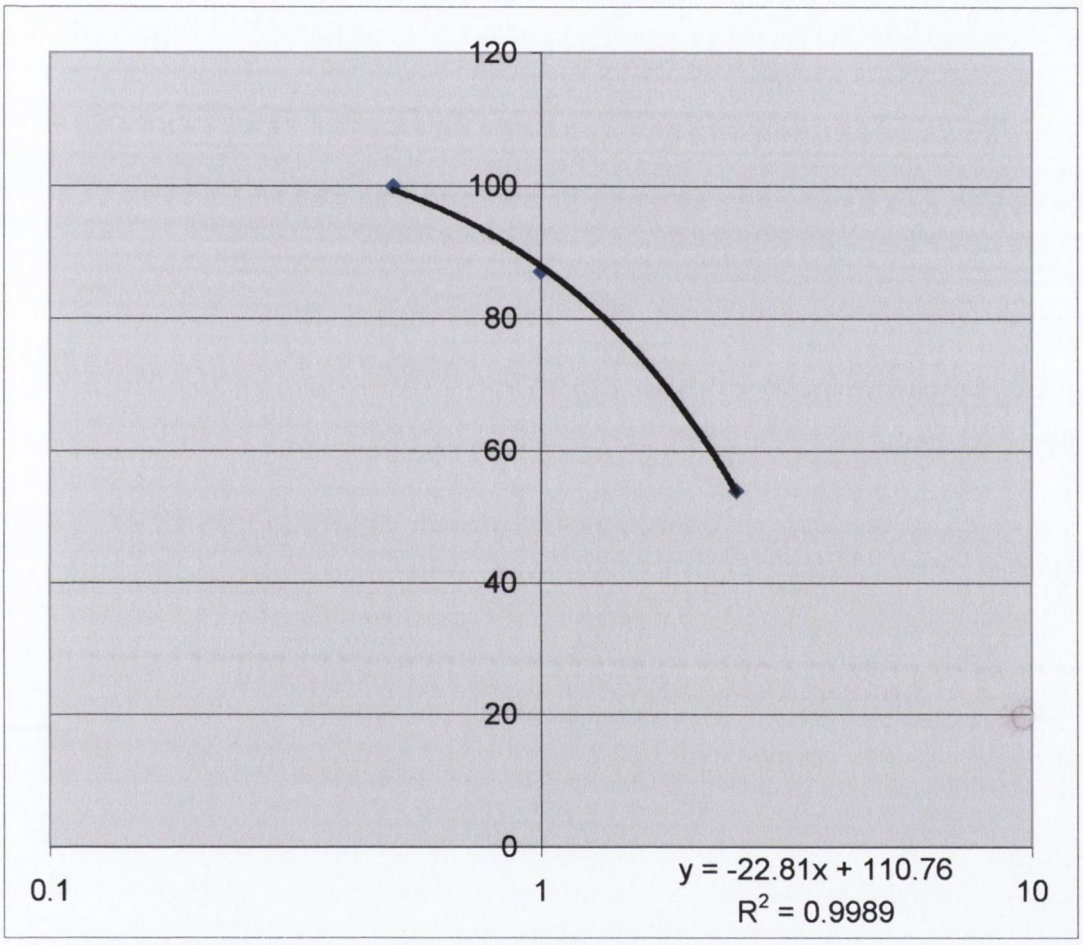
Appendices

Appendix 1 Sample calibration curve for tetracycline base and tetracycline HCl

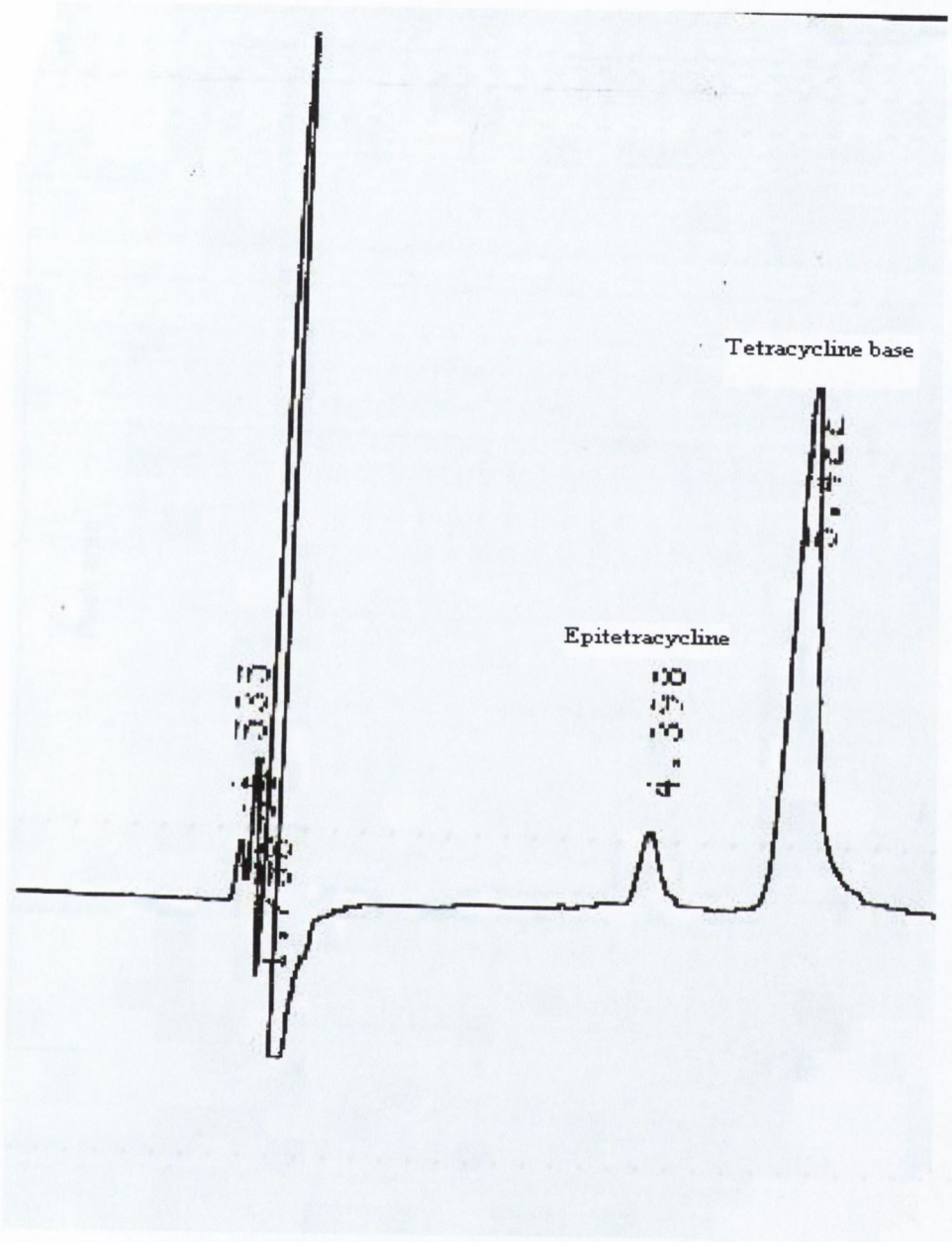




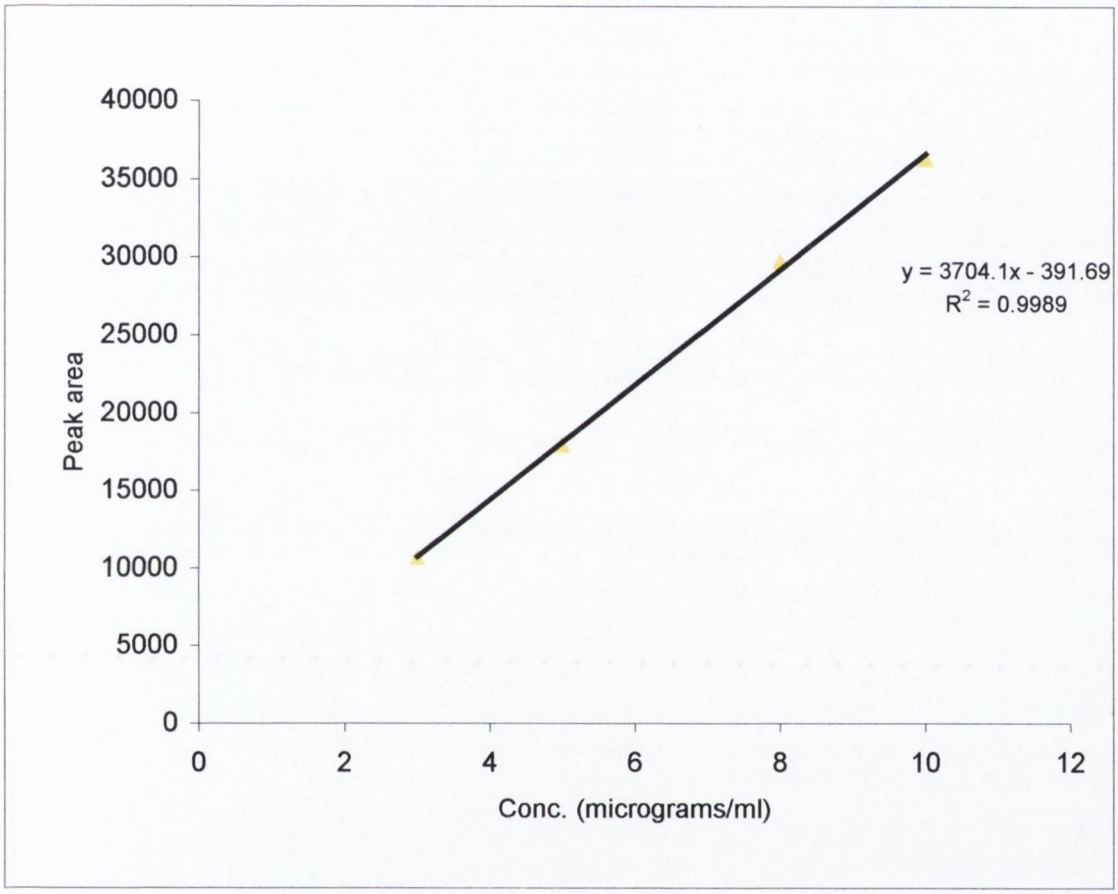
Appendix 2 Calibration curve for Fluoride (ppm)



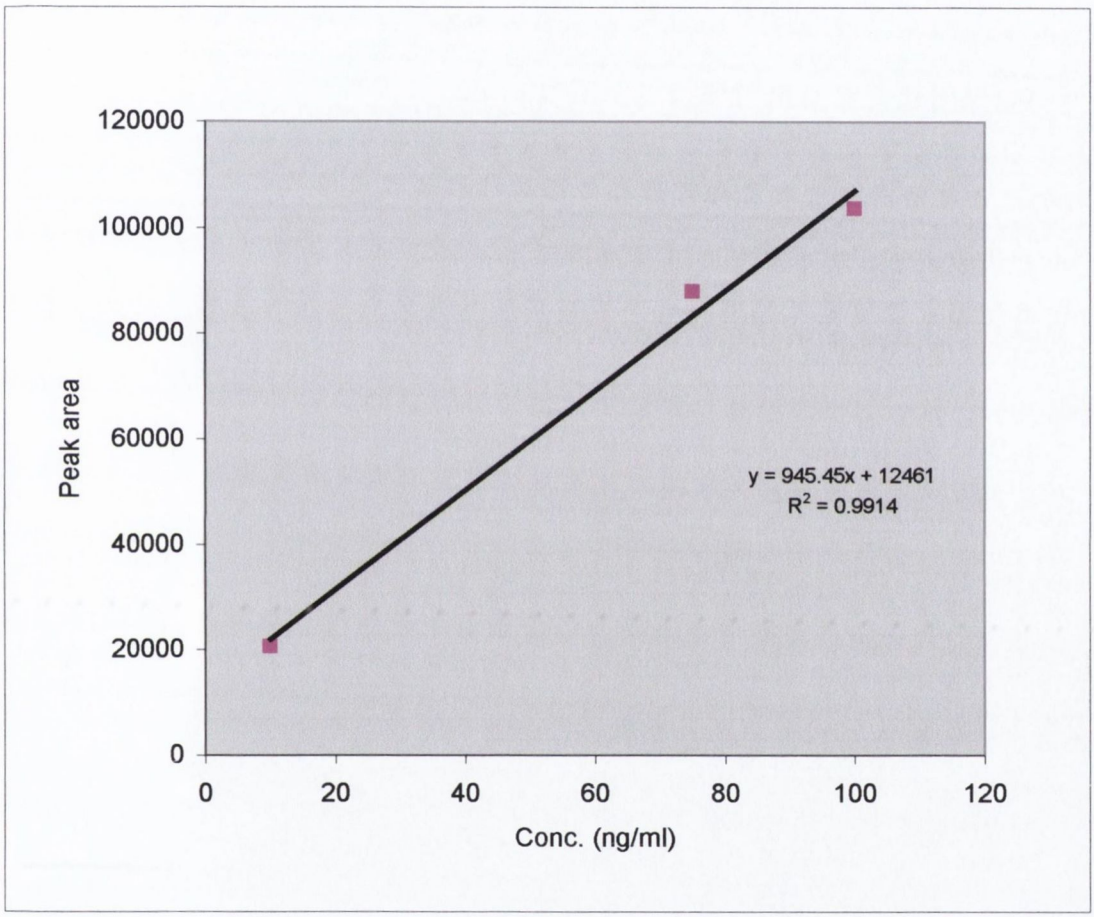
Appendix 3 Sample chromatogram of tetracycline base, obtained using UV detection



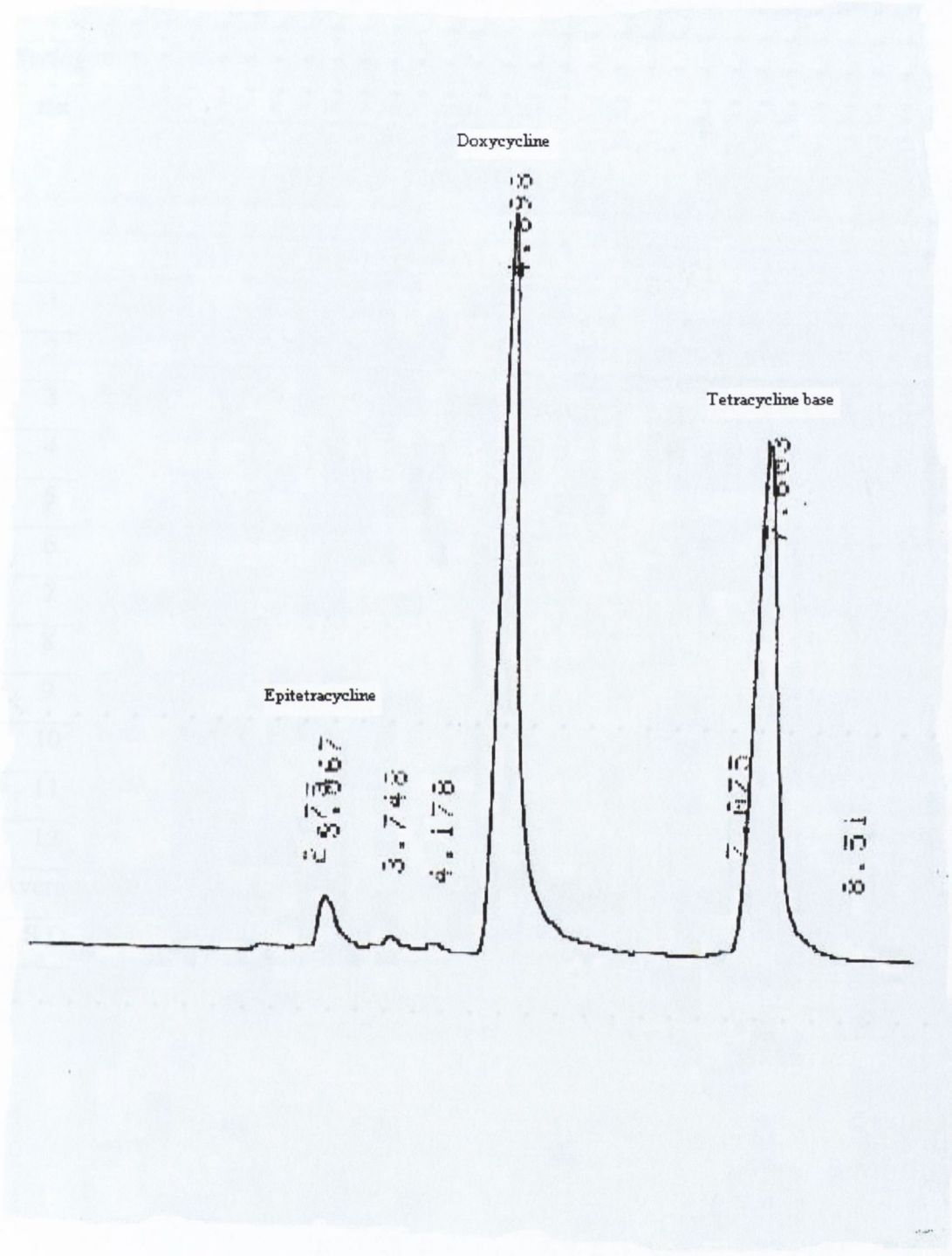
Appendix 4 Sample HPLC calibration curve for tetracycline base using UV detection



Appendix 5 HPLC calibration curve for tetracycline base using fluorescent detection



Appendix 6 Sample chromatogram of tetracycline base and the internal standard, doxycycline, obtained using fluorescent detection



Appendix 7 Weight of samples injected into test and control pockets of dogs

Test product

Syringe no.	*Weight syringe (empty) (g)*	*Weight syringe (filled) (g)*	*Product wt (g)*	*Weight syringe (after injection) (mg)*	*Wt. Prod. injected*	*Periodontal pocket no. (refer to Table 3.7.1 for key)*
1	3.286	4.320	1.034	4.231	0.089	URP2-D1
2	3.294	4.429	1.135	4.362	0.067	LRP2-D1
3	3.310	4.354	1.044	4.124	0.230	LRC-D1
4	3.323	4.333	1.011	4.239	0.095	LRP4-D1
5	3.381	4.105	0.724	4.006	0.098	URC-D1
6	3.332	4.347	1.016	4.199	0.148	URP4-D1
7	3.300	3.975	0.674	3.862	0.112	LLC-D2
8	3.306	4.369	1.063	4.229	0.140	LLP2-D2
9	3.277	4.307	1.030	4.223	0.084	LLP4-D2
10	3.380	4.359	0.980	4.243	0.116	ULC-D2
11	3.323	4.381	1.058	4.302	0.079	ULP2-D2
12	3.327	4.300	0.974	3.899	0.401	ULP4-D2
Average			0.9784		0.1383	
S.D			0.138		0.094	

Control product

Syringe no.	*Weight syringe (empty) (g)*	*Weight syringe (filled) (g)*	*Product wt (g)*	*Weight syringe (after injection) (mg)*	*Wt. Product injected*	*Periodontal pocket no.(refer to Table 3.7.1 for key)*
1C	3.332	4.159	0.827	4.112	0.047	ULP4-D1
2C	3.304	4.416	1.112	4.307	0.110	LLP2-D1
3C	3.372	4.351	0.980	4.253	0.099	LLC-D1
4C	3.309	4.452	1.143	4.245	0.207	LLP4-D1
5C	3.312	3.958	0.647	3.803	0.155	ULC-D1
6C	3.274	4.447	1.173	3.595	0.852	ULP4-D1
7C	3.246	4.137	0.891	4.126	0.012	LRC-D2
8C	3.337	4.403	1.066	4.039	0.365	LRP2-D2
9C	3.334	4.452	1.119	4.227	0.226	LRP4-D2
10C	3.311	4.393	1.082	4.230	0.094	URC-D2
11C	3.281	4.177	0.897	4.137	0.041	URP2-D2
12C	3.339	4.242	0.903	4.138	0.104	URP4-D2
Average			0.987		0.193	
S.D			0.158		0.229	

Appendix 8 Weight of tetracycline residues obtained from sampling pockets

Day 14

Sample no.	*Wt. Container (g)*	*Wt. Container + strips (g)*	*Wt. of container, strips + residue (g)*	*Wt of residue (μg)*	*Pocket (refer to Table 3.7.1 for key)*	*Dog no.*
1	4.730556	4.740080	4.740413	333	LRC	1
2	4.737093	4.746144	4.746873	729	URC	1
3	4.728359	4.737779	4.739408	13629	URP4	2
4	4.752043	4.761500	4.762156	656	LRP2	2
5	4.510025	4.519745	4.50196	451	LRC	2
6	4.705404	4.715098	4.715236	138	LRP4	2
7	4.781621	4.791462	4.791906	444	URP2	2
8	4.527468	4.536964	4.537435	471	URC	2
9	4.52477	4.53403	4.53631	2280	ULP4	2
10	4.71208	4.72215	4.72251	360	LLC	2
11	4.61169	4.62014	4.62175	1610	LLP2	2
12	4.56577	4.57597	4.57604	70	ULP2	2
13	4.72047	4.73044	4.73097	530	ULC	2
14	4.68953	4.69938	4.70031	930	LRP2	1
15	4.76228	4.77176	4.77264	880	LLP4	2
16	4.804581	4.8059	4.80568	590	LRP4	1
17	4.99863	5.00831	5.00883	520	LLC	1
18	4.92802	4.93785	4.93860	750	LLP2	1
19	4.96412	4.97363	4.97528	1650	ULC	1
20	4.91678	4.92607	4.92701	940	URP4	1
21	5.04870	5.05896	5.06041	1450	LLP4	1
22	5.04205	5.05156	5.05233	770	LLP2	1
23	4.97985	4.98968	4.99018	500	LLP4	1
24	4.91207	4.92191	4.92278	870	URP2	1

Day 28

Sample no.	*Wt. of Container (g)*	*Wt. of Container + strips (g)*	*Wt. of container, strips + residue (g)*	*Wt of residue (μg)*	*Pocket (refer to Table 3.7.1 for key)*	*Dog no.*
1	4.573341	4.583107	4.58154	1047	ULP2	2
2	4.504914	4.514405	4.514803	398	LRC	1
3	4.723087	4.732935	4.733456	521	URP4	2
4	4.528301	4.537997	4.538625	628	LLP2	2
5	4.529095	4.538530	4.539883	1353	ULP4	2
6	4.490576	4.599746	4.500514	768	URP2	2
7	4.697677	4.707763	4.708510	747	LLC	2
8	4.577291	4.5866541	4.586913	258.9	LRP4	1
9	4.520009	4.529944	4.530483	539	LLP2	1
10	4.537871	4.547331	4.548020	689	URC	2
11	4.583982	4.593471	4.593834	363	ULP2	1
12	4.517919	4.527268	4.527611	343	URC	1
13	4.652164	4.661950	4.662662	712	ULC	2
14	4.634041	4.643623	4.664012	389	LRP2	1
15	4.633525	4.673642	4.674299	657	URP2	1
16	4.503836	4.513300	4.513824	524	ULC	1
17	4.505498	4.514995	4.515521	526	URP4	1
18	4.578614	4.588211	4.588997	786	LRP2	2
19	4.602765	4.612129	4.612515	386	LLP4	2
20	4.590517	4.600206	4.600480	274	ULP4	1
21	4.556260	4.565904	4.566440	536	LRC	2
22	4.526420	4.536309	4.536516	207	LLC	1
23	4.487197	4.496962	4.49776	814	LRP4	2
24	4.632650	4.642058	4.642385	327	LLP4	1

Day 42

Sample no.	*Wt. of Container (g)*	*Wt. of Container + strips (g)*	*Wt. of container, strips + residue (g)*	*Wt of residue (μg)*	*Pocket (refer to Table 3.7.1 for key)*	*Dog no.*
1	4.52028	4.529984	4.530378	394	LLP2	2
2	4.498608	4.508061	4.508333	272	ULP4	1
3	4.487974	4.497027	4.498223	1196	URP2	1
4	4.532305	4.541436	4.542195	759	LRC	2
5	4.708019	4.717447	4.718483	1036	ULP2	1
6	4.644081	4.653777	4.654015	238	URC	2
7	4.494168	4.498492	4.504524	632	ULC	1
8	4.576621	4.585674	4.586009	335	ULC	2
9	4.678429	4.687450	4.688407	957	URC	1
10	4.536140	4.545853	4.546481	628	LRP2	2
11	4.534200	4.543908	4.544038	130	LLC	1
12	4.656152	4.665755	4.666184	429	URP2	2
13	4.504160	4.513132	4.513697	565	URP4	2
14	4.703666	4.713140	4.713462	322	LLP4	2
15	4.609620	4.618616	4.619392	776	URP4	1
16	4.581971	4.591405	4.591862	457	LLP2	1
17	4.585267	4.595392	4.595454	62	LRP2	1
18	4.561536	4.570835	4.571470	635	LRP4	1
19	4.627656	4.637083	4.637157	74	LLP4	1
20	4.491648	4.501849	4.501794	945	LRC	1
21	4.644002	4.653171	4.654718	1547	LRP4	2
22	4.696541	4.705752	4.706313	561	LLC	2
23	4.512347	4.521978	4.522516	538	ULP4	2
24	4.686115	4.695464	4.696215	751	ULP2	2

Day 56

Sample no.	*Wt of container*	*Wt. Container + strips (g)*	*Wt. f container, strips + residue (g)*	*Wt of residue (μg)*	*Pocket (refer to Table 3.7.1 for key)*	*Dog no.*
1	2.461620	2.471217	2.471658	441	ULC	1
2	2.463311	2.472615	2.483037	422	LRP2	1
3	2.464709	2.473940	2.474223	283	URP4	1
4	2.475884	2.485156	2.485485	329	LLP4	1
5	2.444136	2.453236	2.453745	509	LRC	2
6	2.435319	2.444648	2.444969	321	URC	1
7	2.460313	2.469488	2.470104	616	LLP2	2
8	2.435181	2.443565	2.443754	189	LLC	1
9	2.473186	2.482553	2.483117	564	ULC	2
10	2.478483	2.489260	2.488247	987	URC	2
11	2.473876	2.482982	2.483791	809	ULP2	2
12	2.442584	2.451747	2.451980	233	URP2	2
13	2.419134	2.428308	2.428822	514	URP4	2
14	2.433574	2.443207	2.443274	67	LRP4	2
15	2.444676	2.453928	2.454324	396	ULP4	2
16	2.468923	2.478257	2.478608	351	LRP4	1
17	2.445720	2.455004	2.455492	488	LLP4	2
18	2.471349	2.480712	2.480979	267	ULP4	1
19	2.472793	2.481829	2.482242	413	LRP2	2
20	2.485688	2.495103	2.495701	598	URP2	1
21	2.445525	2.454961	2.455259	298	LLC	2
22	2.504960	2.514371	2.515085	714	LLP2	1
23	2.463575	2.472862	2.473160	298	LRC	1
24	2.461275	2.470322	2.470581	259	ULP2	1

Appendix 9 Microbiological counts for each time point sampled

DAY 0

DOG 1

1.	ULC	40	100
2.	ULP2	20	80
3.	ULP4	50	20
4.	LLC	20	50
5.	LLP2	60	80
6.	LLP4	40	20
7.	URC	160	50
8.	URP2	170	30
9.	URP4	40	60
10.	LRC	20	110
11.	LRP4	50	50
12.	LRP2	30	40

DOG 2

	POCKET	*AEROBIC*	*ANAEROBIC*
1.	ULC	50	80
2.	ULP2	130	100
3.	ULP4	31	100
4.	LLC	150	80
5.	LLP2	100	70
6.	LLP4	70	90
7.	URC	60	70
8.	URP2	100	80
9.	LRC	30	80
10.	LRP2	160	90
11.	LRP4	90	90
12.	URP4	80	110

DAY 14

DOG 1

1.	ULC	CONTROL	610	440
2.	ULP2	CONTROL	980	790
3.	ULP4	CONTROL	860	730
4.	LLC	CONTROL	1050	860
5.	LLP2	CONTROL	320	230
6.	LLP4	CONTROL	470	20
7.	URC	TEST	580	360
8.	URP2	TEST	110	50
9.	URP4	TEST	60	20
10.	LRC	TEST	540	410
11.	LRP4	TEST	200	20
12.	LRP2	TEST	190	40

DOG 2

	POCKET		***AEROBIC***	***ANAEROBIC***
1.	ULC	TEST	110	50
2.	ULP2	TEST	80	10
3.	ULP4	TEST	90	10
4.	LLC	TEST	150	60
5.	LLP2	TEST	180	30
6.	LLP4	TEST	190	50
7.	URC	CONTROL	790	460
8.	URP2	CONTROL	710	70
9.	LRC	CONTROL	1200	1330
10.	LRP2	CONTROL	1640	490
11.	LRP4	CONTROL	210	270
12.	URP4	CONTROL	190	200

DAY 28

DOG 1

1.	ULC	CONTROL	1030	570
2.	ULP2	CONTROL	840	790
3.	ULP4	CONTROL	420	400
4.	LLC	CONTROL	1860	400
5.	LLP2	CONTROL	560	430
6.	LLP4	CONTROL	680	1430
7.	URC	TEST	10	10
8.	URP2	TEST	590	410
9.	URP4	TEST	60	20
10.	LRC	TEST	490	80
11.	LRP4	TEST	90	10
12.	LRP2	TEST	70	10

DOG 2

	POCKET		***AEROBIC***	***ANAEROBIC***
1.	ULC	TEST	110	80
2.	ULP2	TEST	20	10
3.	ULP4	TEST	290	170
4.	LLC	TEST	80	210
5.	LLP2	TEST	470	240
6.	LLP4	TEST	260	230
7.	URC	CONTROL	120	100
8.	URP2	CONTROL	570	240
9.	LRC	CONTROL	870	490
10.	LRP2	CONTROL	470	190
11.	LRP4	CONTROL	930	760
12.	URP4	CONTROL	110	130

DAY 42

DOG 1

1.	ULC	CONTROL	sample lost	
2.	ULP2	CONTROL	730	250
3.	ULP4	CONTROL	40	20
4.	LLC	CONTROL	370	590
5.	LLP2	CONTROL	2300	1200
6.	LLP4	CONTROL	230	280
7.	URC	TEST	210	190
8.	URP2	TEST	180	70
9.	URP4	TEST	150	90
10.	LRC	TEST	50	290
11.	LRP4	TEST	20	30
12.	LRP2	TEST	720	100

DOG 2

	POCKETS		***AEROBIC***	***ANAEROBIC***
1.	ULC	TEST	140	310
2.	ULP2	TEST	320	840
3.	ULP4	TEST	1090	1460
4.	LLC	TEST	470	70
5.	LLP2	TEST	180	40
6.	LLP4	TEST	1240	1070
7.	URC	CONTROL	220	480
8.	URP2	CONTROL	1270	120
9.	LRC	CONTROL	330	490
10.	LRP2	CONTROL	180	40
11.	LRP4	CONTROL	1830	1780
12.	URP4	CONTROL	110	130

12.

DAY 56

DOG 2

	POCKETS		*AEROBIC*	*ANAEROBIC*
1.	ULC	TEST	10	140
2.	ULP2	TEST	10	120
3.	ULP4	TEST	30	340
4.	LLC	TEST	30	30
5.	LLP2	TEST	670	740
6.	LLP4	TEST	280	190
7.	URC	CONTROL	210	630
8.	URP2	CONTROL	850	330
9.	LRC	CONTROL	50	120
10.	LRP2	CONTROL	60	270
11.	LRP4	CONTROL	90	160
12.	URP4	CONTROL	790	860

DOG 1

1.	ULC	CONTROL	50	10
2.	ULP2	CONTROL	10	10
3.	ULP4	CONTROL	10	170
4.	LLC	CONTROL	30	120
5.	LLP2	CONTROL	40	120
6.	LLP4	CONTROL	90	250
7.	URC	TEST	530	90
8.	URP2	TEST	680	90
9.	URP4	TEST	120	50
10.	LRC	TEST	440	170
11.	LRP4	TEST	10	10
12.	LRP2	TEST	10	110

Appendix 10 Probing pocket depths at each time point sampled

DOG 1

1. ULC	5	4	4	4	Control
2. ULP2	2	1	1	1	Control
3. ULP4	2	1	2	1	Control
4. LLC	5	5	4	4	Control
5. LLP2	2	2	2	2	Control
6. LLP4	3	1	2	2	Control
7. URC	4	4	4	4	Test
8. URP2	3	2	1	1	Test
9. URP4	2	2	3	2	Test
10. LRC	4	4	3	3	Test
11. LRP2	2	2	1	2	Test
12. LRP4	2	1	1	2	Test

DOG 2

	DAY 14	*DAY 28*	*DAY 42*	*DAY 56*	
1. ULC	5	4	4	4	Test
2. ULP2	2	1	2	2	Test
3. ULP4	2	2	2	2	Test
4. LLC	4	4	4	4	Test
5. LLP2	2	1	2	2	Test
6. LLP4	2	1	2	1	Test
7. URC	5	4	3	4	Control
8. URP2	2	1	1	1	Control
9. URP4	4	3	2	2	Control
10. LRC	4	4	3	3	Control
11. LRP2	3	2	1	2	Control
12. LRP4	3	2	2	1	Control